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Polyacrylamide Gel Electrophoresis of *Anthonomus grandis* Boheman Proteins

Profile of a Standard Boll Weevil Strain

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Profile of a Standard Boll Weevil Strain

By A. C. Terranova[1]

ABSTRACT

Electrophoretic techniques are presented for an isozyme program in which 1 or 2 trained technicians can study 24 enzyme systems per boll weevil in groups of up to 24 insects per day at a total material cost of about $25 (1979). More than 100 isozymes were detected in the Florence lab strain, a standard boll weevil strain. Of the 35 loci tentatively identified, 60% were polymorphic and 40% were monomorphic. Index terms: *Anthonomus grandis* Boheman, electrophoresis, enzyme systems, isozyme analysis, polyacrylamide gels.

INTRODUCTION

Nearly 30% of all insecticides used in American agriculture are used to control the cotton boll weevil, *Anthonomus grandis* Boheman.[2] As a result, many natural enemies of other pest species are destroyed, which leads to further crop losses, control costs, and environmental contamination (Bottrell 1976). Because of the economic importance of the boll weevil, the U.S. Department of Agriculture in cooperation with State agencies and the National Cotton Council, has launched a massive beltwide pest management offensive against this insect, the ultimate aim of which is eradication.

One aspect of this program involves the mass rearing of boll weevils for sterile male release. Insects mass-reared for mass-release must be compatible and competitive with their native counterparts; but, the objective of most mass-rearing programs is simply to produce the maximum number of insects as inexpensively as possible because there are no known predictive tests for determining the efficiency of insects reared in the laboratory for release. The performance of insects colonized in laboratories and rearing facilities can be impaired by a variety of factors, such as nutritional deficiencies, environmental conditions, disease, improper conditioning and handling before release, and genetic selection during colonization.

Isozyme analysis is ideally suited for detecting and measuring changes in genetic variability attributed to colonization of an insect strain in the laboratory. Once an insect population with desired behavioral traits and genetic makeup is successfully installed in a mass-rearing program, periodic sampling of isozyme allele frequencies in this population may give an early indication of genetic changes.

We are now using electrophoretic techniques to estimate isozyme variability in various laboratory and wild populations of the boll weevil. Literature on isozyme analysis in the boll weevil is scant; therefore, we have incorporated as fully as practical the following requirements set forth by Lewontin

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[2]Coleoptera: Curculionidae.

(1974) to obtain reasonably reliable estimates of genetic variation in populations: (1) A large sample of loci, (2) a varied sample of loci not heavily weighted by a few enzymatic functions, (3) an unbiased sample of loci not chosen consciously or unconsciously because of known variability, (4) enzyme functions that include both those directly involved in energy metabolism and those involved in other vital functions, and (5) a sample of adequate size (at least 50 genomes for each locus).

Quality control and survey programs require precision, reliability, and consideration of three other practical factors: (1) Amount of time needed to obtain results, (2) quantity and type of personnel needed, and (3) expense of materials necessary to maintain an ongoing program. This report presents a complete description of polyacrylamide gel electrophoretic techniques that enable 1 or 2 trained technicians to study 24 enzyme systems per boll weevil in groups of up to 24 boll weevils per day at a total expendable material cost of about $25 (1979) and reports the results of such an analysis of the Florence lab strain of the boll weevil. These results will provide the basis for most of our future work with the boll weevil. The electrophoresis equipment used with the techniques described here was reported by Terranova (1978) unless otherwise noted.

MATERIALS AND METHODS

Insects

The boll weevil colony (Florence lab strain) at the Southeast Cotton Production Research Unit was established in the summer of 1961 with about 200 boll weevils that had emerged from field-collected cotton squares in Florence County, S.C. A microsporidium disease was discovered in the colony in 1966 and was eliminated in the following manner. One hundred pairs of boll weevils were segregated from the colony, and the rest of the colony was destroyed. Eggs were collected from each pair for 2 weeks and seeded in plates containing diet; then the adult females were examined for microsporidia. Only plates with eggs (usually 50 or more) from disease-free females were kept, and these were used to reestablish the colony. When microsporidia were rediscovered in the colony in 1972, the process was repeated.

Diet and rearing conditions have undergone numerous changes during the 18 years in which the colony has been in culture. The basic procedure of Moore et al. (1967) has been followed, except the diet has been modified by the addition of 5% Liquid Cyclone Process cottonseed (Vix et al. 1971) in place of egg albumin. Also, Alphacel and cottonleaf powder have been eliminated.

Boll weevils from the colony used in these analyses were sexed 4 to 6 days after eclosion, killed by quick freezing at −75° C, and stored at −75° C until the analyses were made.

Chemicals

The chemicals used in the preparation of samples, electrophoresis, and isozyme analysis of the 24 boll weevil enzyme systems reported here are listed in table 1. Also listed are the catalog numbers and suppliers of these chemicals.

Gel Formulations

The number of gel formulas available for polyacrylamide gel electrophoresis is extremely large. This is illustrated by the computer program developed by Jovin and Dante (1970), which can generate almost unlimited multiphasic buffer systems, and by the representative output of 4,269 multiphasic buffer systems generated by this program (Jovin et al. 1970). By judicious use of this array of gel formulations, almost any proteins differing in charge or size from each other can be separated. For most of our survey work, however, we must be satisfied with a system that can be managed by one or two people on a daily basis. Two basic formulations have proved adequate for our needs. One is the original formulation of Ornstein (1964) and Davis (1964), which has been, by far, the formulation most used by researchers; the other is the system of Williams and Reisfeld (1964). Table 2 presents the formulas for the stock solutions required to prepare 300 vertical slab gels measuring 83 by 102 by 1.4 mm (enough for 24 gels per day, 3 days per week for 1 month). Table 3 presents the required amounts of these solutions necessary to produce 24 gels.

Gel Preparation

Eight gel formulations were chosen for use with the 24 enzymes normally analyzed (table 4). On the

Table 1.—Chemicals used in study

Chemical	Abbreviation, formula, or symbol	Source[1]	Catalog number
GEL PREPARATION			
Acrylamide	ACR	Bio-Rad	161-0100
Ammonium persulfate	AmPer	Bio-Rad	161-0700
N,N'-Methylene-bis-acrylamide	BIS	Bio-Rad	161-0200
Riboflavin	RIBO	Bio-Rad	161-0500
Riboflavin-5′-phosphate	R-5-P	Bio-Rad	161-0501
Sucrose	SUC	Bio-Rad	161-0720
N,N,N',N'-Tetramethylenediamine	TEMED	Bio-Rad	161-0800
BUFFER PREPARATION			
Boric acid	H_3BO_4	Sigma	B 0252
Citric acid	CIT	Sigma	C 7129
5,5′-Diethylbarbituric acid	BARB	Sigma	B 0375
Glycine	GLY	Sigma	G 7126
Hydrochloric acid	HCl	Baker	9535
Maleic acid	MA	Sigma	M 0375
Phosphoric acid	H_3PO_4	Baker	2-0260
Potassium phosphate, monobasic	KH_2PO_4	Baker	3246
Sodium hydroxide	NaOH	Baker	3722
Sodium phosphate, monobasic	NaH_2PO_4	Baker	3818
Sodium phosphate, dibasic	Na_2HPO_4	Baker	3822
Tris(hydroxymethyl)aminomethane	TRIS	Sigma	T 1503
ENZYME SUBSTRATES			
L-Aspartic acid	ASP	Sigma	A 9256
Benzaldehyde	BEN	Baker	9144
DL-β(3,4-Dihydroxyphenyl)-alanine	DOPA	Sigma	D 9503
D-Fructose-6-phosphate	F-6-P	Sigma	F 3627
Fumaric acid	FUM A	Sigma	F 4502
α-D(+) Glucose	GLU	Sigma	G 5000
α-D-Glucose-1-phosphate	G-1-P	Sigma	G 7000
D-Glucose-6-phosphate	G-6-P	Sigma	G 7879
DL-α-Glycerophosphate	α-GLY	Sigma	G 6126
1-Hexanol	HEX	Baker	1-9307
Hypoxanthine	HYP	Sigma	H 9377
DL-Isocitric acid	ISO	Sigma	I 1252
α-Ketoglutaric acid	KETO	Sigma	K 1750
DL-Lactic acid	LAC	Sigma	L 1375
L-Leucyl-β-naphthylamide	LBN	Sigma	L 0376
DL-Malic acid	MAL	Sigma	M 0875
α-Naphthyl acetate	α-NAP	Sigma	N 6750
β-Naphthyl acetate	β-NAP	Sigma	N 6875
α-Naphthyl acid phosphate	NAP	Sigma	N 7000
1-Octanol	OCT	Sigma	O 4500
2-Propanol	PROP	Baker	9084
Starch, soluble	STA	Sigma	S 2630
Tyrosine	TYR	Sigma	T 3379
BIOCHEMICALS			
Adenosine-5-diphosphate	ADP	Sigma	A 0127
Adenosine-5-triphosphate	ATP	Sigma	A 3127

See footnote at end of table.

Table 1.—Chemicals used in study—Continued

Chemical	Abbreviation, formula, or symbol	Source[1]	Catalog number
BIOCHEMICALS—CONTINUED			
Albumin, bovine	ALB	Sigma	A 4378
Bromophenol blue	BPB	Bio-Rad	161-0404
Coomassie brilliant blue R-250	COOM	Bio-Rad	161-0400
Fast black K salt	FBK	K&K	16318
Fast blue BB salt	FBBB	Sigma	F 0250
Fast blue RR salt	FBRR	Sigma	F 0500
Fast garnet GBC salt	GBC	Sigma	F 0875
Myoglobin	MYO	Sigma	M 0630
β-Nicotinamide adenine dinucleotide	NAD	Sigma	N 7004
Nicotinamide adenine dinucleotide phosphate	TPN	Sigma	N 0505
Nitro-blue tetrazolium	NBT	Sigma	N 6876
Phenazine methosulfate	PMS	Sigma	P 9625
Polyvinyl pyrrolidone	PVP	Sigma	PVP-40
Pyridoxyl-5′-phosphate	PYR	Sigma	P 9255
ENZYMES			
Glucose-6-phosphate dehydrogenase	G-6-PD	Sigma	G 7878
Hexokinase	HK	Sigma	H 4502
Malic dehydrogenase	MDH	Sigma	410-12
MISCELLANEOUS			
Acetic acid, glacial	HAC	Baker	3-9507
Acetone	ACET	Baker	3-9006
Calcium chloride	$CaCl_2$	Baker	1-1308
Iodine, sublimed	I	Baker	4-2208
Magnesium chloride	$MgCl_2$	Sigma	M 0250
Manganous chloride	$MnCl_2$	Baker	1-2540
1-Propanol	1-PROP	Baker	9086
Potassium iodide	KI	Sigma	P 8256
Sodium chloride	NaCl	Baker	1-3624
Trichloroacetic acid	TCA	Baker	1-0414

[1]Main addresses:
Bio-Rad Laboratories, 2200 Wright Ave., Richmond, Calif. 94804.
J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg, N.J. 08865.
K&K Laboratories, 121 Express St., Plainsview, N.Y. 11803.
Sigma Chemical Co., P. O. Box 14508, St. Louis, Mo. 63178.

Table 2.—Stock solutions for polyacrylamide gel preparation[1]

Stock solution	Final pH	Final volume with distilled water (ml)	Components[2]										
			1*N* HCl (ml)	TRIS (g)	TEMED (ml)	1*M* H_3PO_4 (ml)	ACR (g)	BIS (g)	RIBO[3] (g)	SUC (g)	AmPer (g)	GLY (g)	BARB (g)
A1	8.9	750	180	137.25	0.90	...	.....	....	....	...	...	...	.....
A2	7.5	150	36	5.14	.18	...	.....	....	....	...	...	...	.....
B1	6.7	250	120	14.95	1.15	...	.....	....	....	...	...	...	.....
B2	5.5	100	...	4.96	.46	39	.....	....	....	...	...	...	.....
C5	...	150	...	.....	....	...	29.00	1.00	....	...	...	...	.....
C7	...	650	...	.....	....	...	175.83	6.06	....	...	...	...	.....
C9	...	150	...	.....	....	...	52.16	1.80	....	...	...	...	.....
D	...	150	...	.....	....	...	42.00	1.12	....	...	...	...	.....
E	...	300	...	.....	....	...	.....	....	.012	...	...	...	.....
F[4]	...	150	...	.....	....	...	.....	....	....	60	...	...	.....
G[4]	...	450	...	.....	....	...	.....	....	....	...	.63	...	.....
H1[5]	8.3	20,000	...	120.00	....	...	.....	....	....	...	...	576	.....
H2[6]	7.0	20,000	...	20.00	....	...	.....	....	....	...	...	...	110.4

[1]Solutions stored at 4° C in the dark.
[2]See table 1 for names of chemical components.
[3]Use riboflavin-5′phosphate for formula 2 gels (0.008 g/100 ml).
[4]Prepare fresh each week.
[5]To use, dilute 1:9; upper and lower buffer for formula 1 gels; discard after use.
[6]To use, dilute 1:1; upper and lower electrode buffer for formula 2 gels; discard after use.

day before electrophoresis, gels were prepared in two stages. The required amounts of stock solutions were brought to polymerization temperature (20° C) in the preparation bath (Terranova 1978). Once these solutions (see separating gels, table 3) had attained the proper temperature, 12 cells were placed in the bath and checked for leaks. The appropriate A and C separating gel solutions were mixed and degassed, as was solution G, with a water aspirator. Solution G was then mixed carefully with the other two solutions to avoid introduction of air. Because of the speed of polymerization of the mixture, the completed separating gel solution was pipetted to a depth of 65 mm in each of the 12 cells within 5 minutes. A free-flowing water-layering syringe containing 1.0 ml of distilled water was attached to the top of each cell and left undisturbed until the gel polymerized. Unpolymerized liquid was carefully removed from the top of each cell by suction. Each gel was rinsed twice with the appropriate solution (table 3). The room was then darkened, and the appropriate stacking-well forming gel components (table 3) were mixed and pipetted on top of the separating gel. The sample well formers, each containing 24 wells, were put in place, and the gels were allowed to photopolymerize for at least 30 minutes. After polymerization, the 12 gels were placed into their respective electrophoresis chambers and stored overnight at 5° C. The second group of 12 gels was then prepared in exactly the same way.

Sample Preparation

Boll weevil adults were removed from the freezer and immediately homogenized individually in an ice bath for 30 seconds in 1.5-ml microtest tubes. Each tube contained 250 μl of a solution of 20% sucrose in distilled water. The homogenates were centrifuged at 4° C for 20 minutes at 10,000 × g. The resulting supernatants were transferred to clean microtest tubes and recentrifuged at 10,000 × g for 10 minutes. These supernatants were either used immediately or were frozen at −75° C until electrophoresis the next day. Before use, 10 μl of undiluted stock A1 containing 0.05% bromophenol blue (BPB) was added to 50-μl aliquots of each sample contained in wells of a Cooke Microtiter Plate held in an ice bath. In addition, 5 μl and 20 μl of stocks B2 and B1, both containing 0.5% BPB, were added to 25- and 100-μl aliquots, respectively, of each sample. From 2 to 10 μl of each sample was applied to one slot in each of the appropriate vertical-slab gels, depending on the enzyme system being tested (table 4). The remaining sample, about

Table 3.—Working solutions for preparation of polyacrylamide gels for 24 boll weevil enzyme systems

Separating gels								Rinsing solutions							Stacking-well forming gels							
Gel formula[2]	No. gels	Stock solutions (ml)[1]						Gel formula[2]	No. gels	Stock solutions (ml)[1]					Gel formula[2]	No. gels	Stock solutions (ml)[1]					
		A1	A2	C5	C7	C9	G			A1	B1	B2	E	Water			A1	B1	B2	D	E	Water	F
1.5A	1	3	...	3	...	...	6	1A	7	3	...	...	3	18	1A	6	3	...	...	3	3	3	12
1.7A	6	18	...	...	18	...	36	1	13	...	6	...	6	36	1	14	...	6	...	6	6	6	24
1.5	2	6	...	6	...	...	12	2	4	...	...	2	2	12	2	4	...	...	2	2	2	2	8
1.7	10	30	...	...	30	...	60	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1.9	1	3	...	...	...	3	6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2.5	1	...	3	3	...	...	6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2.7	1	...	3	...	3	...	6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2.9	2	...	6	...	...	6	12	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

[1]Refer to table 2.

[2]Whole number 1 refers to formulation of Ornstein (1964) and Davis (1964). Whole number 2 refers to formulation of Williams and Reisfeld (1964). Fractional number refers to $\%T$ (total concentration of both acrylamide and BIS).

Table 4.—Enzyme systems studied in the boll weevil

Enzyme	Abbreviation	Gel formula used[1]	Sample applied[2] (μl)	Total cost per gel[3]
Acid phosphatase	AP	2.9	2	$0.84
Alkaline phosphatase	ALK P	2.9	2	.83
Alcohol dehydrogenase (ADH):[4]				
Hexanol dehydrogenase	HEX DH	1.7	6	1.05
Octanol dehydrogenase	ODH	1.7	6	1.04
2-Propanol dehydrogenase	2-prop. DH	1.7	10	.94
Aldehyde oxidase	AO	1.5	2	.81
Adenylate kinase	AK	1.7	10	1.48
Amylase	AMY	1.9	10	.60
Esterase	EST	1.7	6	.74
Fumerase	FUM	1.7A	10	1.05
Glutamate-oxaloacetate transaminase	GOT	2.5	10	.81
α-Glycerophosphate dehydrogenase	α-GPD	1.7A	6	.91
Glucose-6-phosphate dehydrogenase	G-6-PD	1.7A	2	1.36
Hexokinase	HK	1.7	10	1.38
Isocitrate dehydrogenase[5]	IDH	1.5	6	2.42
Lactate dehydrogenase	LDH	1.7A	10	1.05
Leucine aminopeptidase	LAP	2.7	6	.90
Malate dehydrogenase	MDH	1.7A	2	1.05
Malic enzyme	ME	1.7A	6	1.23
Phosphoglucomutase	PGM	1.7	6	1.74
Phosphoglucose isomerase	PGI	1.7	6	1.30
Tetrazolium oxidase	TO	1.7	6	.87
Tyrosinase	TYR	1.7	10	.83
Xanthine dehydrogenase	XDH	1.5A	10	1.13

[1]From table 3.

[2]From total homogenate volume of 250 μl.

[3]Includes cost of disposable cells at $0.40 each, gel components at $0.14 each, and electrode buffers at $0.04 each for formula 1 gels; $0.24 each for formula 2 gels; and stain components from table 6 (based on April 1979 prices).

[4]Add 1 mg NAD to unpolymerized gel components; add 1 mg NAD to upper buffer before run.

[5]Add 1 mg TPN to unpolymerized gel components; add 1 mg TPN to upper buffer before run.

75 μl, was refrozen for use in other tests, such as total protein determinations, isoelectric focusing, or perhaps rerunning an enzyme system.

Standard Preparation

The reference standards, 10 mg of myoglobin and 30 mg of bovine serum albumin, were dissolved in 5 ml of distilled water containing 20% sucrose, and 200-μl aliquots were stored frozen at −75° C. Before use, undiluted A1, B1, or B2, all containing 0.5% BPB, were added to 50-, 100-, and 25-μl portions of the standard, respectively. Five μl of the appropriate standard mixture was applied to gel slots 1 and 13 of each gel during sample application. These standards served as a check of the electrophoresis procedures.

Electrophoresis

On the morning after gel preparation, each of the 24 lower gel baths was filled with 600 ml of the appropriately diluted lower buffer (table 2), which had previously been chilled to 5° C. The bottom seal was removed from each of the gel cells, and the cells were replaced in position in the upper baths. After all 24 gels were in place, 300 ml of the appropriate upper buffer (table 2) was poured into the gel baths, and the sample well formers were removed. Using a 10-ml syringe, each sample well was rinsed with upper buffer to remove unpolymerized gel components. In groups of 4 gels, the samples were applied to the appropriate wells, the electrode leads were attached, and the bath complexes were installed on the support stations

of the multifunctional electrophoresis system (Terranova 1978).

A constant current of 10 mA was applied to each of the gels for about 30 minutes until the BPB tracking dye entered the separating gel. The current was then adjusted to 30 mA per gel until the tracking dye had migrated the desired distance (usually 55 mm) into the separating gel. As electrophoresis in each group of 4 gels was completed, power was discontinued to that group, and the gels were removed from the cells and treated according to the appropriate procedure for the enzyme. When the first group (gels 1-4) was finished, it was removed and replaced with gels 13-16. The same routine was followed with each of the remaining 2 sets of 4 gels until all 24 were finished. Thus, while some gels were electrophoresing, others were being developed for a particular enzyme. Electrophoresis of all 24 gels was accomplished in 4.5 to 5.0 hours.

Isozyme Development

In many laboratories (Brewer 1970, Shaw and Prasad 1970, Gabriel 1971, Bush and Huettel 1972, and Steiner and Joslyn 1979) stock solutions of many of the individual components necessary for enzyme staining are prepared and then stored for extended periods. These are mixed in the proper amounts just before gel development. This is an excellent time-saving technique when only a few systems are being analyzed or when sufficient labor is available. However, it becomes a time-consuming and confusing chore in a laboratory that daily analyzes 24 systems that require mixing of more than 50 solutions in various combinations. This is especially so when only one or two individuals are responsible for the total electrophoresis procedure.

The following procedure was developed to alleviate this problem. Using the appropriate buffer (table 5), the various components listed in tables 6 and 7 for each enzyme system were premixed or preweighed once a month, according to instructions in the footnotes, in sufficient quantities to accomodate development of the anticipated number of gels to be electrophoresed during the month. Each of the prepared stock solutions from table 6 was divided into a sufficient number of aliquots for electrophoresis each day and immediately frozen and stored at −75° C. The reagents from table 7, which were added to the premixed solutions in table 6 just before gel development, were likewise prepared in sufficient quantities and stored as directed.

Normally, as each group of 4 gels was placed in the electrophoresis unit, the corresponding staining solutions were taken from the freezer and placed in a drawer beneath the workbench. After the gels had been electrophoresed, the length of the separating gel, the migration distance of the tracking dye, and the migration distance of the protein standards were measured, and the remaining components were added. The gels were then removed from the glass cells and treated with the thawed staining mixtures.

For development, each gel slab was placed between two 102- by 152-mm sheets of plastic (such as that found in many commercially available sandwich bags). This was a departure from the method presented earlier (Terranova 1978). Three sides of the sheets were sealed together with a plastic bag sealer to form a bag containing the gel, leaving one side open. Then, 10 ml of the appropriate enzyme-staining mixture was poured in, and the remaining side was sealed. The bag was then placed in a 102- by 127- by 0.64-mm porous plastic box (available at many film finishing outlets) and attached to a rotary temperature bath, as previously discussed (Terranova 1978). The reaction time at 37° C normally ranged from 30 to 60 minutes, depending on the enzyme system (table 7). After the gels had been developed to the desired intensity, they were removed from the bags, rinsed with water, and fixed in 50 ml of 7% acetic acid solution for at least 30 minutes.

This procedure considerably reduced the time required for daily preparation of stain solutions, and the amounts of chemicals used for gel development for each of the 24 enzyme systems in this study represented only 10% to 20% of the amounts recommended in the original recipes. Many of the chemicals are expensive; thus, this constituted a tremendous savings (table 4).

Permanent Gel Records

Once the gels had become fixed, each was duplicated on a permanent, full-size diazo transparency in which each isozyme appeared as a black band against a transparent background. First, 100 sheets of 216- by 279-mm GAF black diazo color film (102 V) were cut to 102 by 127 mm and stored in a dark container at 5° C. Just before use, 24

(*Continued on page 13.*)

Table 5.—Buffers for preparation of staining solutions

No.	Name	Preparation	Dilution for use	Reference
1	TRIS-HCl	98.8 g TRIS, 25 ml concentrated HCl, pH 8.4; make 2 l with distilled water.	1:3	Bush and Huettel (1972).
2	Phosphate	54.4 g KH_2PO_4, 240+ ml of 1.0 *M* NaOH, pH 7.4; make 1,000 ml with distilled water.	1:3	Bush and Huettel (1972).
3	Phosphate	(A) 27.6 g of 0.2 *M* NaH_2PO_4 in 1,000 ml distilled water, pH 6.5. (B) 71.7 g of 0.2 *M* $Na_2HPO_4 \cdot 12H_2O$ in 1,000 ml distilled water, pH 6.5.	A:B:H_2O (5:1:4)	Shaw and Koen (1969).
4	TRIS-maleate	(A) 24.2 g TRIS, 23.2 g of MA (0.2 *M* TRIS acid maleate, pH 3.8) in 1,000 ml distilled water. (B) 8.0 g of 0.2 *M* NaOH in 1,000 ml distilled water.	LAP (pH 5.2) A:B:H_2O (5:1:4) AP (pH 6.0) A:B:H_2O (2:1:5)	Bush and Huettel (1972).
5	TRIS-CIT	18.42 g TRIS, 2.10 g CIT in 2,000 ml distilled water.	1:1	Poulik (1957).
6	TRIS-HCl	17.44 g TRIS, 1:1 HCl, pH 8.0, in 1,000 ml distilled water.	None	McKechnie et al. (1975).
7	Boric acid, 0.5 *M*	30.92 g H_3BO_4, 1.02 g $MgCl_2$ in 1,000 ml distilled water.	None	Brewer (1970).
8	TRIS-HCl	17.44 g TRIS, 1:1 HCl (pH 7.4), 2.0 g $CaCl_2$ in 1,000 ml distilled water.	None	Doane (1967).

Table 6.—Composition of premixed components of enzyme-staining solutions

Gel No.	Enzyme	Buffer No.[1]	Substrate			$MgCl_2$[2] (mg)	$MnCl_2$[2] (mg)	NaCl[3] (mg)	PVP[2] (mg)	1-PROP (ml)	ADP (mg)	ATP (mg)	NAD[4] (mg)	TPN[5] (mg)
			Chemical	Amount: Milliliters	Amount: Grams									
1	AO	1	BEN	5.0										
2	TO	1				200								
3	ADH	1	PROP	5.0									25	
4	ODH	1	OCT	5.0									25	
5	HEX DH	1	HEX	5.0									25	
6	α-GPD	1	α-GLY		1.0								25	
7	LDH	1	LAC	10.0									25	
8	XDH	1	HYP[6]		1.0								25	
9	MDH	1	MAL		1.0	100							25	
10	FUM	1	FUM A		1.0								25	
11	EST	3	α-NAP[7]											
			β-NAP[7]											
12	AMY	8	STA[8]		.5									
13	ME	1	MAL		1.0	100								50
14	G-6-PD	1	G-6-P		.2	100								50
15	PGM	1	G-1-P		1.5	100								50
			G-1-P, G-6-P		.002									
16	PGI	1	F-6-P		.025	100								50
17	IDH	6	ISO		1.7	500								100
18	TYR	3	DOPA		.1									
			TYR		.1									
19	GOT	2	ASP		.5									
			KETO		.05									
			PYR		.02									
20	HK	1	GLU		1.0	100							50	50
21	AK	1	GLU		1.0	100						50		50
22	LAP	4	LBN		.1									
23	ALK P	5	NAP		.15	100	100	300	100					
24	AP	4	NAP		.15	100	100	300	100					

[1]Dissolve components in buffer and add water to bring to 100 ml; check pH and adjust if necessary; store up to 60 days at −75° C in 10-ml aliquots. Buffer numbers correspond to those in table 5.

[2]Dissolve 10 g in 100 ml distilled water; 1.0 ml contains 100 mg.

[3]Dissolve 30 g in 100 ml distilled water; 1.0 ml contains 300 mg.

[4]Store dessicated at −20° C in 10-ml glass vials holding 225 mg each; just before use dissolve in 9 ml distilled water; 1.0 ml contains 25 mg.

[5]Store dessicated at −20° C in 10-ml glass vials holding 450 mg each; just before use dissolve in 8 ml distilled water; 1.0 ml contains 50 mg.

[6]Add 0.1 g to each of 10 vials with 10 ml buffer; heat to dissolve just before using; let cool and add remaining reagents; use 0.1 ml of NAD (footnote 4) per test.

[7]Dissolve 200 mg each of α-NAP and β-NAP in 10 ml acetone and store at −20° C; add 1.0 ml to staining buffer; 1.0 ml contains 20 mg of each component.

[8]Boil 5 minutes before measuring and freezing aliquots.

Table 7.—Components of enzyme-staining solutions added to 10-ml aliquots just before gel development

Gel No.	Enzyme	NBT[1] (mg)	PMS[2] (mg)	Enzyme solution (units)			GBC[6] (mg)	FBK[6] (mg)	FBRR[6] (mg)	FBBB[6] (mg)	Iodine solution[7] (ml)	Development time (min)	Comments
				G-6-PD[3]	HK[4]	MDH[5]							
1	AO	2.0	0.5	...	...	....	...	...	...	...	...	30	
2	TO	4.0	1.0	...	...	....	...	...	...	...	...	60	Expose to light for development of colorless bands on blue background.
3	ADH	2.0	.5	...	...	....	...	...	...	...	...	60	
4	ODH	2.0	.5	...	...	....	...	...	...	...	...	60	
5	HEX DH	2.0	.5	...	...	....	...	...	...	...	...	60	
6	*a*-GPD	2.0	.5	...	...	....	...	...	...	...	...	60	
7	LDH	2.0	.5	...	...	....	...	...	...	...	...	60	
8	XDH	2.0	.5	...	...	....	...	...	...	...	...	60	
9	MDH	2.0	.5	...	...	....	...	...	...	...	..	60	
10	FUM	2.0	.5	...	...	37.5	...	...	...	...	...	60	
11	EST	...	...	...	...	....	5.0	...	...	...	...	45	Preincubate at 5° C in buffer 3; change buffer twice every 15 min.
12	AMY	...	...	...	...	....	...	...	...	...	25	3	Incubate 60 min in buffer 8 at 37° C before adding iodine.
13	ME	2.0	.5	...	...	....	...	...	...	...	...	30	
14	G-6-PD	2.0	.5	...	...	....	...	...	...	...	...	30	
15	PGM	2.0	.5	10	...	....	...	...	...	...	...	60	
16	PGI	2.0	.5	10	...	....	...	...	...	...	...	60	
17	TYR	...	...	...	...	....	...	...	...	...	...	240	Activate gel in 1:1 ETOH:buffer 3 at 5° C for 2 h; wash gel 30 min in 3 changes buffer 3; add substrate solution and incubate at 37° C.

See footnotes at end of table.

Table 7.—Components of enzyme-staining solutions added to 10-ml aliquots just before gel development—Continued

Gel No.	Enzyme	NBT[1] (mg)	PMS[2] (mg)	Enzyme solution (units)			GBC[6] (mg)	FBK[6] (mg)	FBRR[6] (mg)	FBBB[6] (mg)	Iodine solution[7] (ml)	Development time (min)	Comments
				G-6-PD[3]	HK[4]	MDH[5]							
18	IDH	2.0	.5	...	...	....	...	...	...	...	...	60	Preincubate at 5° C in buffer 6; change buffer twice every 15 min.
19	GOT	...	...	...	...	....	...	...	...	5.0	...	60	Preincubate at 5° C in buffer 2; change buffer twice every 15 min.
20	HK	2.0	.5	10	...	....	...	...	...	...	...	60	
21	AK	2.0	.5	10	20	....	...	...	...	...	...	60	
22	LAP	...	...	...	...	....	...	5.0	...	...	...	60	Preincubate at 5° C in buffer 7; change buffer twice every 15 min.
23	ALK P	...	...	...	...	....	...	...	5.0	...	...	30	Preincubate at 5° C in buffer 5 for 15 min.
24	AP	...	...	...	...	....	...	...	5.0	...	...	30	Preincubate at 5° C in buffer 4; change buffer 4 times every 15 min.

[1]Store desiccated at 4° C in 5-ml glass vials holding 40 mg each; just before use dissolve in 2.0 ml distilled water; 0.1 ml contains 2.0 mg.
[2]Store desiccated at 4° C in 5-ml glass vials holding 10 mg each; just before use dissolve in 2.0 ml distilled water; 0.1 ml contains 0.5 mg.
[3]100 units per vial; add 1.0 ml distilled water and store at −20° C; 0.1 ml contains 10 units.
[4]200 units per vial; add 1.0 ml distilled water and store at −20° C; 0.1 ml contains 20 units.
[5]750 units per vial; bring volume to 1.0 ml (with water) and store at 5° C; 0.05 ml contains 37.5 units.
[6]Store desiccated at −20° C in gelatin capsules containing 5.0 mg each.
[7]Dissolve 30 g KI and 13 g I in 1,000 ml distilled water; store at room temperature.

sheets were removed and allowed to warm to room temperature in subdued light. A black, felt-tip pen was used to record date, enzyme system, sample, etc., on the top 12.8 mm of the coated side of the film. After the ink had dried (30 to 60 seconds), each film was slipped into a 102- by 152-mm plastic bag (catalog No. BB561, Bolab Inc., 6 Tinkham Ave., Derry, N.H. 03038) and placed coated-side-up onto a platform located 330 mm below two Ken Rad 275-W sun lamps (Elder and Agee 1977). The corresponding gels were drained and placed on top of the bags, directly in line with the film; care was taken to exclude all air bubbles. The films were exposed to the sun lamps for 2 to 3 minutes and then removed from the plastic bags and developed for 60 seconds in a Tupperware sandwich box containing about 100 ml of undiluted household ammonia (Elder and Agee 1977). All 24 transparencies were developed in the same solution. The ink washed off, but the data and isozyme patterns were permanently recorded on the film. After drying, the films were replaced in the 102- by 152-mm bags along with a 102- by 152-mm file card that was used to record the gel data, and the whole packet was stored in a file cabinet of appropriate size. Bands were measured either from the diazo transparencies or from the gels themselves.

Identification of Isozyme Positions

Johnson (1971) showed the importance of having some measure of the variations in band position attributed to experimental conditions (variations in temperature, pH, ionic strength, gel concentration, etc.) versus those attributed to intrinsic differences between proteins (size, shape, or charge). This is especially true in enzyme surveys of natural insect populations or in quality control of laboratory insect populations, in which studies are often conducted over a period of years. Since the mobility of a protein under a given set of electrophoretic conditions is a physical constant (Rodbard and Chrambach 1974), the inclusion of two protein standards in at least two slot positions of every gel served as a check of the ambient factors. Moreover, as Johnson (1971) showed, the ratios between the standards and between the standards and the proteins of interest are more accurate for the determination of protein variation than is the measure of their mobility in relation to the running front (Rf) alone.

The results of this study were calculated as follows: First, the ratio between the migration distances of the myoglobin and albumin standards was calculated for each gel to determine electrophoretic reproducibility. Then the value of each protein band (isozyme) was calculated as the ratio between its migration distance and that of either myoglobin (Rmyo) or albumin (Ralb) or of the tetrazolium oxidase 1.10 band (Rto) found in most systems in which the nitro-blue tetrazolium (NBT) and phenazine methosulfate (PMS) reagents were used.

Myoglobin and albumin were chosen as standards because their paths through the gel could be followed visually during electrophoresis. Myoglobin appeared as a reddish-brown spot, and albumin complexed with the BPB tracking dye and appeared as a blue spot. The distance of travel of these standards and the tracking dye was measured immediately after electrophoresis because subsequent treatment of the gels usually causes a loss of color in these areas. The front of the tracking dye was further marked with a notch in the gel at either end of the front because the dye is usually eluted during the enzyme development procedures. The position of the protein standards, however, could be seen again by light staining of the fixed gels with Coomassie blue after enzyme development.

The tetrazolium oxidase (TO) 1.10 band was chosen as a standard because it proved to be invariant in all boll weevil strains tested. After confirmation of its mobility relative to the myoglobin standard, the TO 1.10 band served as an excellent visual internal standard in most systems in which the NBT and PMS reagents were used.

In preliminary tests, each enzyme system was tested for completeness of reaction as follows: Two gels containing identical aliquots of boll weevil extract were electrophoresed according to the procedure for each enzyme system. One gel was developed in 100 ml of the original staining recipe made fresh just before development, and the other gel was developed in 10 ml of the modified recipe as outlined. No differences in isozyme content or stain intensity could be detected for any of the systems.

Each enzyme system was then tested for artifactual bands by deleting the substrates from one 10-ml aliquot and developing identical gels with and without substrate. This produced no banding in any of the gels developed in solutions devoid of substrate, except that in a few of the dehydrogenase gels very faint, usually indistinct bands were sometimes observed; these apparently corresponded to those found in alcohol dehydrogenase

(ADH) (fig. 3). Other unidentified bands were present just below the TO 1.10 band in some dehydrogenase gels (figs. 13 and 15). These bands were sporadic in appearance and not scorable and may have corresponded to alcohol dehydrogenase activity, since traces of ethanol resulting from the manufacturing process usually remain in some of the histochemicals.

The effect of storage on the staining solutions was determined with three identical gels that were treated with freshly prepared, 30-day-old, and 60-day-old frozen aliquots of the modified stain solutions. There was no difference between the fresh and 30-day-old solutions, and little difference was noted with the 60-day-old solutions, which did show more background staining and somewhat lighter isozyme bands for some systems. We therefore adopted the policy of using the premixed solutions within about 30 days.

RESULTS AND DISCUSSION

Most of these techniques and procedures were adaptations of those used in polyacrylamide gel electrophoresis, and the instructions represented a protocol that was deemed necessary to routinely electrophorese 24 gels in a single day. The modifications in buffer, pH, gel concentrations, etc., were those found to be necessary through trial and error to obtain reproducible results with the biological material used or to insure compatibility with the equipment reported earlier (Terranova 1978).

Sample Preparation

Generally, the fresher the sample, the better the result. Our best results with regard to isozyme activity, band definition, and reproducibility of banding patterns were with insects processed within 1 month after sacrifice. However, when large populations were to be studied, this was not always possible. Our experience has shown, in all but a few cases, that boll weevils frozen whole at −75° C change little in isozyme content and definition for as long as about 1 year in storage. However, isozyme activity for most systems is lowered at freezer temperatures of about −20° C. Once an insect has been sacrificed, that insect and its homogenates must be kept as cold as possible during subsequent operations. Many of the enzyme systems lose activity within a few hours at room temperature. Thus, when working with the homogenates, one should keep them in an ice bath while applying samples to the gels. Also, if the remainder of the homogenates are to be kept, they should be returned as soon as possible to the freezer and stored at −75° C. Such samples should be used within a few days.

Although a critical study was not conducted, the enzyme systems that deteriorated most with prolonged storage were acid phosphatase (AP) and alkaline phosphatase (ALK P), which lost definition; in the case of AP, isozyme 2.58 was completely lost within 4 months (within a few days in homogenized samples). Alpha-glycerophosphate dehydrogenase (α-GPD) and lactate dehydrogenase (LDH) also deteriorated rapidly, within a few months, in the Florence lab strain. The rest of the enzyme systems remained essentially unchanged after prolonged storage at −75° C.

The 20% sucrose homogenates of boll weevils were directly electrophoresed with good results, but treatment of aliquots with stock solutions A1, B1, and B2, as outlined, seemed to result in a little better resolution of the isozymes, especially in boll weevils that had been in storage for prolonged periods.

Gel Preparation

Gels were normally prepared at 20° C the day before use and stored overnight at 5° C. None of the gels separated from the glass sides, nor did the separating characteristics of the gels differ from those of freshly prepared gels.

Standardization

Table 8 shows the amount of variation in migration distances and mobility rations of two protein standards within a single gel, within a group of gels run simultaneously and made from the same batch of gel components, and from different gel runs with different batches of reagents for the 1.7 gel formula. Although the error associated with measuring actual migration distance of a protein or its mobility relative to a standard is not greatly different within a gel or within a group of gels run at the same time, there is clearly a significant advantage in using the mobility ratio between two internal standards rather than the actual migration distance or the ratio of migration of a protein relative to the front. This especially applies to long-range

Table 8.—Variation in migration distances and mobility ratios of two protein standards within a single gel, within a group of gels run simultaneously and made from the same batch of gel components, and from different gel runs with different batches of reagents for the 1.7 gel formula

Sample size	Statistic[1]	Migration distance			Mobility ratio		
		Front	Myoglobin	Albumin	Myo/front	Alb/front	Myo/alb
		WITHIN A SINGLE GEL					
[2]24	Mean	57.2	27.7	44.4	0.49	0.78	0.63
	SD	0.4	0.3	0.3	0.006	0.006	0.004
	CV	0.7	1.1	0.7	1.2	0.8	0.7
	SE	0.08	0.06	0.06	0.001	0.001	0.001
		WITHIN A GROUP OF GELS					
[3]24	Mean	59.1	28.5	45.9	0.48	0.78	0.62
	SD	0.7	0.6	0.8	0.009	0.01	0.008
	CV	1.1	2.1	1.8	1.8	1.2	1.2
	SE	0.14	0.12	0.16	0.002	0.002	0.002
		DIFFERENT GROUPS					
[4]75	Mean	57.2	26.9	43.1	0.47	0.75	0.63
	SD	1.6	1.9	3.3	0.03	0.06	0.02
	CV	2.7	6.9	7.6	6.6	7.6	3.5
	SE	0.2	0.2	0.4	0.004	0.007	0.003

[1]Means for migration distances in millimeters. SD=Standard deviation. CV=Coefficient of variation (%). SE=Standard error.

[2]1 gel containing 24 protein standard replicates.

[3]24 separate gels; migration distances determined from slot 13 standards.

[4]75 separate gels, representing 10 different batches of gel components; migration distances determined from slot 13 standards.

Table 9.—Variation in mobility ratios of myoglobin and albumin protein standards within a single gel and from different gels representing different reagents for each of the gel formulations used in study

Gel formula	Myoglobin/albumin ratios					
	Within gel variation			Between gel variation		
	No. gels[1]	Mean	CV[2]	No. gels[3]	Mean	CV[2]
1.5A	1	0.56	0.9	6	0.56	1.6
1.5	1	.57	.6	44	.55	3.6
1.7A	1	.62	.8	.42	.61	3.6
1.7	1	.62	.6	75	.63	3.5
1.9	1	.70	.5	19	.69	2.3
2.5	1	.79	.5	16	.77	2.2
2.7	1	.66	.5	17	.64	3.3
2.9	1	.53	.9	22	.52	2.7

[1]Each gel contains 24 protein standard replicates.

[2]CV=Coefficient of variation (%).

[3]Numerous gels made from at least 5 different batches of gel components; mobility ratios determined from slot 13 standards.

comparisons involving either different batches of components or homogenates on different gels that may have been run months or even years apart, as often happens during population studies. According to Johnson (1971), "Once the mobility ratios of two or more proteins have been determined and the limits of the variation in experimental procedure have been established, the mobility of a protein variant may be characterized by the ratio of its movement to that of a standard, and any differences in the ratio of variant protein to standard must be due to a difference in the intrinsic nature of the variant. Also, if the ratio of distances moved by the internal markers is different from normal, the differences may be used to correct the mobility values of experimental bands."

Table 9 shows the variation in mobility ratios of the myoglobin and albumin standards for each of the gel formulations used in this study, and figure 19 shows the variation in mobility ratios of the TO 1.10 band in those gels for which it served as a standard.

Isozymes

Although the Florence lab strain of boll weevil has been reared in the laboratory for 18 years, the colony, at least genetically, cannot be considered 18 years old because it was reestablished in 1966 and again in 1972. Some of the original heterogeneity may have been lost through founder effects precipitated by the microsporidium outbreaks and subsequent selection. Thus, the present gene pool of the colony may be considerably different than when the colony was originally established. However, since the reestablishment of this strain in 1972, there have apparently been no further selection pressures; thus, it was assumed that this strain mates randomly.

A descriptive discussion of each enzyme system is presented to show the results obtained with the methods employed. In the absence of inheritance data, conformation of electrophoretic patterns of simple genetic models and chi-square tests for fit to Hardy-Weinberg proportions were taken as evidence for the genetic interpretations of the variable loci. Where no variability existed for a particular enzyme system, a single gene coding for the banding pattern was postulated except where obvious multiple zones existed. Isozymes that could not be made to fit genetic models were not used in any of the statistical calculations.

For each locus, the mobility position of each isozyme was given relative to albumin (ALB), myoglobin (MYO) or TO, and in one case to the BPB front (F). The reference point used for each system is designated in parentheses after each subheading. Table 4 gives the abbreviation for the enzyme systems. The legends to the illustrations (figs. 1-21) include the number of observations ($\bar{X}$), standard deviation (S), coefficient of variation (CV), and standard error (SE).

Acid phosphatase and alkaline phosphatase (MYO)

Both AP and ALK P provided identical zymograms, except for one additional AP isozyme present in all individuals tested (fig. 1). Isozymes 1.43 and 1.67 were broad, intense staining bands which tended to coalesce, and females usually stained more intensely than males. However, this intense staining reaction was unique for this lab-reared strain, since native boll weevil collections show much less activity in this area and band resolution is generally better. Phenotypically, either one or both isozymes were present in an individual. These isozymes may represent a locus consisting of two codominant alleles controlling a monomeric protein.

With the Hardy-Weinberg rule (Snyder and David 1957), isozymes 1.43 and 1.67 represent alleles that yielded combined male and female gene frequency values of 0.491 and 0.509, respectively, with an expected heterozygote proportion of 0.500. Evaluation by chi-square yielded a value of 0.34 and a $P > 0.80$ (table 10).

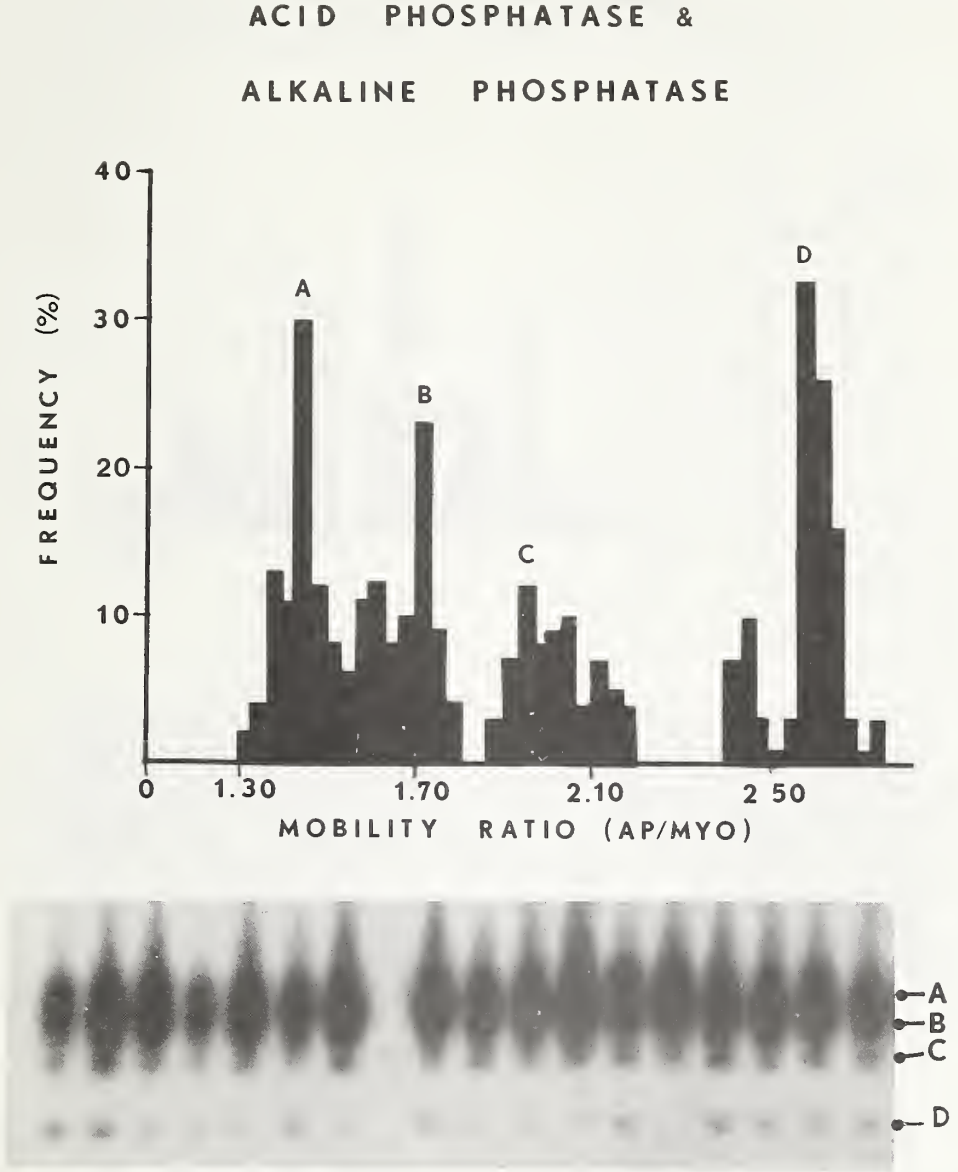
Isozyme 2.02 was a weaker staining isozyme that may or may not be easily detectable in an individual because of the staining intensity of the previously mentioned bands. This electromorph is monomorphic in this boll weevil strain. In other boll weevil strains, however, two bands of activity are readily seen.

Isozyme 2.58, which was detectable only in the AP treated gels, was a moderately staining isozyme present in every individual and presumably represents a monomorphic locus.

Adenylate kinase (F)

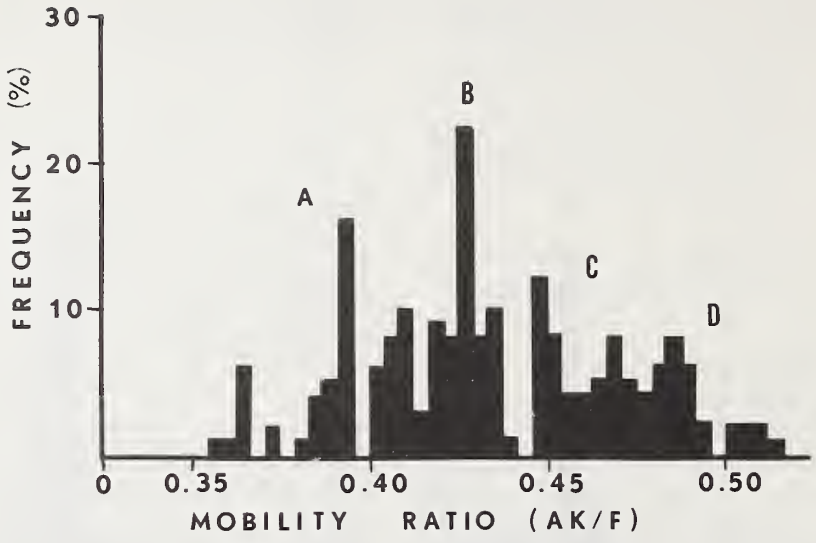
Although the AK enzyme system produced zymograms with apparently good isozyme resolution (fig. 2), a genetic model explaining the observed patterns was not determined. The difficulty with interpreta-

(*Continued on page 24.*)



ISOZYME	N	MOBILITY RATIO (AP/MYO) X	S	CV	SE	RANGE
A	80	1.43	0.046	3.18	0.005	1.30-1.53
B	86	1.67	0.065	3.91	0.008	1.52-1.79
C	69	2.02	0.083	4.12	0.010	1.89-2.20
D	106	2.58	0.078	3.04	0.008	2.42-2.76

FIGURE 1.—Distribution of mobility ratios for acid phosphatase and alkaline phosphatase isozymes from 106 adult boll weevils. Peak D is present in AP only.



		MOBILITY RATIO (AK/F)				
ISOZYME	N	X	S	CV	SE	RANGE
A	36	0.38	0.013	3.26	0.002	0.36-0.39
B	84	0.42	0.010	2.36	0.002	0.40-0.44
C	51	0.46	0.009	2.11	0.002	0.44-0.48
D	28	0.50	0.008	1.58	0.003	0.48-0.51

FIGURE 2.—Distribution of mobility ratios for adenylate kinase isozymes from 84 adult boll weevils.

Table 10.—Allele (electromorph) frequencies, observed heterozygosity, and expected heterozygosity of enzyme loci of the Florence lab strain of boll weevil

Locus	Analysis	Results per given number of insects		
		54 males	52 females	Combined population (106 insects)
AP, ALK P	Allele frequency:			
	Isozyme 1.43	0.537	0.442	0.491
	Isozyme 1.67	0.463	0.558	0.509
	Heterozygote proportion:			
	Observed	0.444	0.615	0.528
	Expected	0.497	0.493	0.500
	Chi-square value (x^2)	0.62	3.17	0.34
	Probability (P)	>0.70	>0.20	>0.80
AP, ALK P	Allele frequency, isozyme 2.02	1.000	1.000	1.000
AP	Allele frequency, isozyme 2.58	1.000	1.000	1.000
		81 males	78 females	Combined population (159 insects)
ADH	Allele frequency, isozyme 0.53	1.000	1.000	1.000
ADH	Allele frequency:			
	Isozyme 0.63	0.673	0.641	0.657
	Isozyme 0.72	0.327	0.359	0.343
	Heterozygote proportion:			
	Observed	0.457	0.410	0.434
	Expected	0.440	0.460	0.451
	Chi-square value (x^2)	0.12	0.91	0.21
	Probability (P)	>0.90	>0.50	=0.90
		48 males	43 females	Combined population (91 insects)
AO	Allele frequency:			
	Isozyme 0.38	0.479	0.512	0.495
	Isozyme 0.43	0.521	0.488	0.505
	Heterozygote proportion:			
	Observed	0.375	0.465	0.418
	Expected	0.499	0.500	0.500
	Chi-square value (x^2)	2.99	0.21	2.47
	Probability (P)	>0.20	=0.90	>0.20
		48 males	49 females	Combined population (97 insects)
AO	Allele frequency:			
	Isozyme 0.67	0.594	0.714	0.670
	Isozyme 0.76	0.406	0.286	0.330
	Heterozygote proportion:			
	Observed	0.521	0.408	0.515
	Expected	0.482	0.408	0.442
	Chi-square value (x^2)	0.31	0.0001	2.68
	Probability (P)	>0.80	>0.99	>0.20

Table 10.—Allele (electromorph) frequencies, observed heterozygosity, and expected heterozygosity of enzyme loci of the Florence lab strain of boll weevil—Continued

Locus	Analysis	Results per given number of insects		
		42 males	42 females	Combined population (84 insects)
AMY	Allele frequency, isozyme 1.11	1.000	1.000	1.000
AMY	Allele frequency:			
	Isozyme 1.25	0.464	0.345	0.405
	Isozyme 1.30	0.536	0.655	0.595
	Heterozygote proportion:			
	Observed	0.595	0.548	0.571
	Expected	0.497	0.452	0.482
	Chi-square value (x^2)	1.62	1.87	2.90
	Probability (P)	>0.30	>0.30	>0.20
		72 males	72 females	Combined population (144 insects)
EST	Allele frequency:			
	Isozyme 0.79	0.028	0.056	0.042
	Isozyme 0.89	0.972	0.944	0.958
	Heterozygote proportion:			
	Observed	0.056	0.111	0.042
	Expected	0.054	0.106	0.080
	Chi-square value (x^2)	0.08	0.23	0.31
	Probability (P)	>0.95	>0.80	>0.80
		61 males	70 females	Combined population (131 insects)
EST	Allele frequency:			
	Isozyme 1.06	0.164	0.264	0.218
	Isozyme 1.13	0.836	0.736	0.782
	Heterozygote proportion:			
	Observed	0.262	0.443	0.359
	Expected	0.274	0.389	0.341
	Chi-square value (x^2)	0.11	1.36	0.40
	Probability (P)	>0.90	>0.50	>0.80
		72 males	72 females	Combined population (144 insects)
EST	Allele frequency:			
	Isozyme 1.40	0.215	0.215	0.215
	Isozyme 1.43	0.215	0.125	0.170
	Isozyme 1.50	0.035	0.049	0.042
	Isozyme 1.65	0.500	0.576	0.538
	Isozyme 1.72	0.035	0.035	0.035
	Heterozygote proportion:			
	Observed	0.709	0.695	0.703
	Expected	0.654	0.602	0.632
	Chi-square value (x^2)	5.61	8.35	8.55
	Probability (P)	>0.95	>0.80	>0.80

Table 10.—Allele (electromorph) frequencies, observed heterozygosity, and expected heterozygosity of enzyme loci of the Florence lab strain of boll weevil—Continued

Locus	Analysis	Results per given number of insects		
		55 males	50 females	Combined population (105 insects)
FUM	Allele frequency, isozyme 0.41	1.000	1.000	1.000
FUM	Allele frequency:			
	Isozyme 0.58	0.891	0.760	0.829
	Isozyme 0.71	0.109	0.240	0.171
	Heterozygote proportion:			
	Observed	0.218	0.400	0.305
	Expected	0.194	0.365	0.285
	Chi-square value (x^2)	0.84	0.46	0.56
	Probability (P)	>0.50	>0.70	>0.70
		51 males	56 females	Combined population (107 insects)
G-6-PD	Allele frequency, isozyme 0.56	1.000	1.000	1.000
		54 males	54 females	Combined population (108 insects)
GOT	Allele frequency:			
	Isozyme 0.39	0	0.009	0.005
	Isozyme 0.53	1.000	0.991	0.995
	Heterozygote proportion:			
	Observed	0	0.019	0.009
	Expected	0	0.018	0.010
	Chi-square value (x^2)	....	0.01	0.01
	Probability (P)	....	>0.99	>0.99
		50 males	48 females	Combined population (98 insects)
α-GPD	Allele frequency:			
	Isozyme 1.03	0.340	0.344	0.342
	Isozyme 1.12	0.660	0.656	0.658
	Heterozygote proportion:			
	Observed	0.480	0.563	0.520
	Expected	0.449	0.453	0.450
	Chi-square value (x^2)	0.24	2.91	2.40
	Probability (P)	>0.80	>0.20	>0.30
		41 males	40 females	Combined population (81 insects)
IDH	Allele frequency:			
	Isozyme 0.70	0.878	0.913	0.895
	Isozyme 0.82	0.122	0.087	0.105
	Heterozygote proportion:			
	Observed	0.195	0.175	0.185
	Expected	0.214	0.159	0.188
	Chi-square value (x^2)	0.31	0.39	0.02
	Probability (P)	>0.80	>0.80	=0.99
	Allele frequency, isozyme 1.20	1.000	1.000	1.000

Table 10.—Allele (electromorph) frequencies, observed heterozygosity, and expected heterozygosity of enzyme loci of the Florence lab strain of boll weevil—Continued

Locus	Analysis	Results per given number of insects		
		54 males	52 females	Combined population (106 insects)
LAP	Allele frequency:			
	Isozyme 0.21	1.000	1.000	1.000
	Isozyme 0.39	1.000	1.000	1.000
LAP	Allele frequency:			
	Isozyme 1.00	0.972	0.952	0.967
	Isozyme 1.14	0.028	0.048	0.033
	Heterozygote proportion:			
	Observed	0.056	0.096	0.066
	Expected	0.054	0.091	0.064
	Chi-square value (x^2)	0.04	0.04	0.12
	Probability (P)	=0.98	=0.98	>0.90
LAP	Allele frequency, isozyme 1.68	1.000	1.000	1.000
		54 males	53 females	Combined population (107 insects)
MDH	Allele frequency:			
	Isozyme 0.58	0.778	0.868	0.822
	Isozyme 0.70	0.222	0.132	0.178
	Heterozygote proportion:			
	Observed	0.407	0.264	0.336
	Expected	0.347	0.229	0.291
	Chi-square value (x^2)	1.71	1.23	2.48
	Probability (P)	>0.30	>0.50	>0.20
		54 males	50 females	Combined population (104 insects)
ME	Allele frequency, isozyme 0.35	1.000	1.000	1.000
ME	Allele frequency:			
	Isozyme 0.56	0.685	0.640	0.664
	Isozyme 0.68	0.315	0.360	0.337
	Heterozygote proportion:			
	Observed	0.407	0.400	0.404
	Expected	0.432	0.461	0.448
	Chi-square value (x^2)	0.17	0.87	0.96
	Probability (P)	>0.90	>0.50	>0.50
		55 males	53 females	Combined population (108 insects)
PGM	Allele frequency:			
	Isozyme 0.65	0.345	0.340	0.343
	Isozyme 0.75	0.382	0.340	0.361
	Isozyme 0.83	0.273	0.320	0.296
	Heterozygote proportion:			
	Observed	0.655	0.736	0.694
	Expected	0.661	0.667	0.665
	Chi-square value (x^2)	0.09	1.83	0.58
	Probability (P)	>0.99	>0.80	>0.98

Table 10.—Allele (electromorph) frequencies, observed heterozygosity, and expected heterozygosity of enzyme loci of the Florence lab strain of boll weevil—Continued

Locus	Analysis	Results per given number of insects		
		43 males	44 females	Combined population (87 insects)
PGI	Allele frequency:			
	Isozyme 0.50	0.500	0.296	0.396
	Isozyme 0.57	0.500	0.704	0.604
	Heterozygote proportion:			
	Observed	0.349	0.364	0.356
	Expected	0.500	0.417	0.478
	Chi-square value (x^2)	3.75	0.70	5.71
	Probability (P)	>0.10	>0.70	>0.10
		54 males	52 females	Combined population (106 insects)
PGI	Allele frequency, isozyme 0.66	1.000	1.000	1.000
		50 males	50 females	Combined population (100 insects)
TO	Allele frequency, isozyme 1.10	1.000	1.000	1.000
		36 males	33 females	Combined population (69 insects)
TYR	Allele frequency:			
	Isozyme 0.25	0.653	0.576	0.616
	Isozyme 0.28	0.347	0.424	0.384
	Heterozygote proportion:			
	Observed	0.361	0.485	0.420
	Expected	0.454	0.405	0.465
	Chi-square value (x^2)	1.48	0.52	0.75
	Probability (P)	>0.30	>0.70	>0.50
		45 males	46 females	Combined population (91 insects)
TYR	Allele frequency:			
	Isozyme 1.04	0.422	0.402	0.412
	Isozyme 1.10	0.578	0.598	0.588
	Heterozygote proportion:			
	Observed	0.444	0.630	0.538
	Expected	0.488	0.481	0.485
	Chi-square value (x^2)	0.36	4.44	1.12
	Probability (P)	>0.80	>0.10	>0.50

Table 10.—Allele (electromorph) frequencies, observed heterozygosity, and expected heterozygosity of enzyme loci of the Florence lab strain of boll weevil—Continued

Locus	Analysis	Results per given number of insects		
		52 males	53 females	Combined population (105 insects)
XDH	Allele frequency:			
	Isozyme 0.39	0.663	0.472	0.567
	Isozyme 0.45	0.337	0.528	0.433
	Heterozygote proportion:			
	Observed	0.519	0.340	0.429
	Expected	0.447	0.498	0.491
	Chi-square value (x^2)	1.39	5.35	1.73
	Probability (P)	>0.50	>0.05	>0.30
		Average, all males	Average, all females	Average, combined population
Frequency of heterozygotes per locus:				
	Observed	0.224	0.248	0.236
	Standard error	0.040	0.043	0.041
Percentage of monomorphic loci		42.9	40.0	40.0

tion of this system suggests that presence of at least some of the isozymes may be attributable to the differential expression of conformers in different individuals or to other nongenetic artifacts. Formal inheritance studies are clearly warranted to determine the nature of this system. However, it appears that this locus was heterozygous.

Alcohol dehydrogenase (TO)

Three isozymes showed ADH activity (fig. 3). Similar patterns were produced when 2-propanol, hexanol, or octanol were used as substrate. However, 2-propanol exhibited the lightest staining bands and octanol the heaviest. Isozyme 0.53 was present in all boll weevils tested and in some samples appeared as two coalesced bands. Isozymes 0.63 and 0.72 were not present in all individuals. Interpretation of the banding patterns revealed that isozyme 0.53 was, in fact, composed of two bands that had the same mobility. One was considered a monomorphic locus, since it was present in every individual; the other corresponded to an allele of another locus that was inferred to be present or absent in some cases, depending on the disposition of the other two isozymes (0.63 and 0.72). If all three bands were present, a heterozygous individual was indicated. If only isozymes 0.53 and 0.72 were evident, an individual homozygous for the 0.72 allele was indicated. However, if only isozyme 0.53 was present, an individual homozygous for the 0.53 allele was indicated. Conversely, some individuals could be directly scored as possessing two bands at the 0.53 point because of the slight separation that sometimes occurs.

When the data were subjected to goodness-of-fit to expectations based on Hardy-Weinberg equilibrium, a chi-square value of 0.21, equal to a P of 90%, was obtained. Thus, this interpretation was accepted as a likely genetic explanation of the observed banding patterns for these alcohol dehydrogenases.

Since all three alcohol substrates gave essentially the same results, it was presumed that the same genes were responsible for 2-prop. DH, HEX DH, and ODH activity because of the nonspecific nature of these enzymes.

Aldehyde oxidase (MYO)

Aldehyde oxidase activity appeared at two separate locations (fig. 4). Isozymes 0.38 and 0.43 stained lightly and lay very close together, which

made them difficult to score. Both isozymes were apparently encoded by the same locus, and individuals produced either one or two bands. In the heterozygotes, most individuals produced a diffuse, broad band. Measurement to the middle of this band usually produced an Rmyo midway between the isozymes.

Isozymes 0.67 and 0.76 produced intensely staining bands that appeared singly or in combination. However, when in combination, a third isozyme (0.71) was always present. This indicated a protein of the dimeric type, encoded by two alleles in which subunits randomly combined. Table 10 gives the gene frequency data associated with these two loci.

Amylase (ALB)

Amylase was composed of two loci. Isozyme 1.11 comprised one locus. In appearance, 1.11 was strongly active and was present in every individual tested, so it was considered monomorphic (fig. 5).

Isozymes 1.25 and 1.30 comprised the second AMY locus. The heterozygotes were composed of these two isozymes, and they usually appeared as a single, broad band of activity. Chi-square evaluation indicates that this population was in Hardy-Weinberg equilibrium (table 10).

Esterase (MYO)

The esterases of the boll weevil, as with many other organisms, constituted the most complex isozyme system of these studies. More than 27 isozymes with esterase activity have been observed in the Florence lab boll weevil strain. Many of these, however, were low in frequency and sporadic in their presence. All but the major isozymes were disregarded in this study. The upper half of the esterase gels (see legend in figure 6) usually contained isozymes of low activity and low frequency. Three polymorphic loci were identified but were not pursued further because null alleles were suspected. Inheritance tests are being conducted to determine the genetics of this area.

The lower half of the gels contained most of the medium-to-intense staining bands, and three variable loci were evident in this area. Isozymes 0.79 and 0.89 were designated as alleles of one locus; isozymes 1.06 and 1.13 were designated as alleles of a second locus; and isozymes 1.40, 1.43, 1.50, 1.65, and 1.72 were designated as alleles of the third locus (B and K, figure 6, were subsequently found to be composed of two distinct isozymes with mean values of 1.40 and 1.43, respectively). In all three loci, homozygous individuals had only one band of activity, and heterozygotes had two bands; no three-banded individuals were observed. Thus, it was presumed that these esterases were monomeric enzymes. These three loci conformed to this genetic interpretation as evidenced by goodness-of-fit to Hardy-Weinberg equilibrium (table 10).

Fumarase (TO)

Two loci were apparent in individuals stained for FUM activity. The first, which was monomorphic, provided a broad, rather fuzzy, dense band at Rto 0.41 (fig. 7). The second FUM locus, characterized by isozymes 0.58 and 0.71, stained lightly but was distinct. The homozygotes displayed a one-banded pattern, whereas the heterozygotes displayed a three-banded pattern consisting of the two homodimers and an intermediate heterodimer of Rto 0.65.

Glucose-6-phosphate dehydrogenase (MYO)

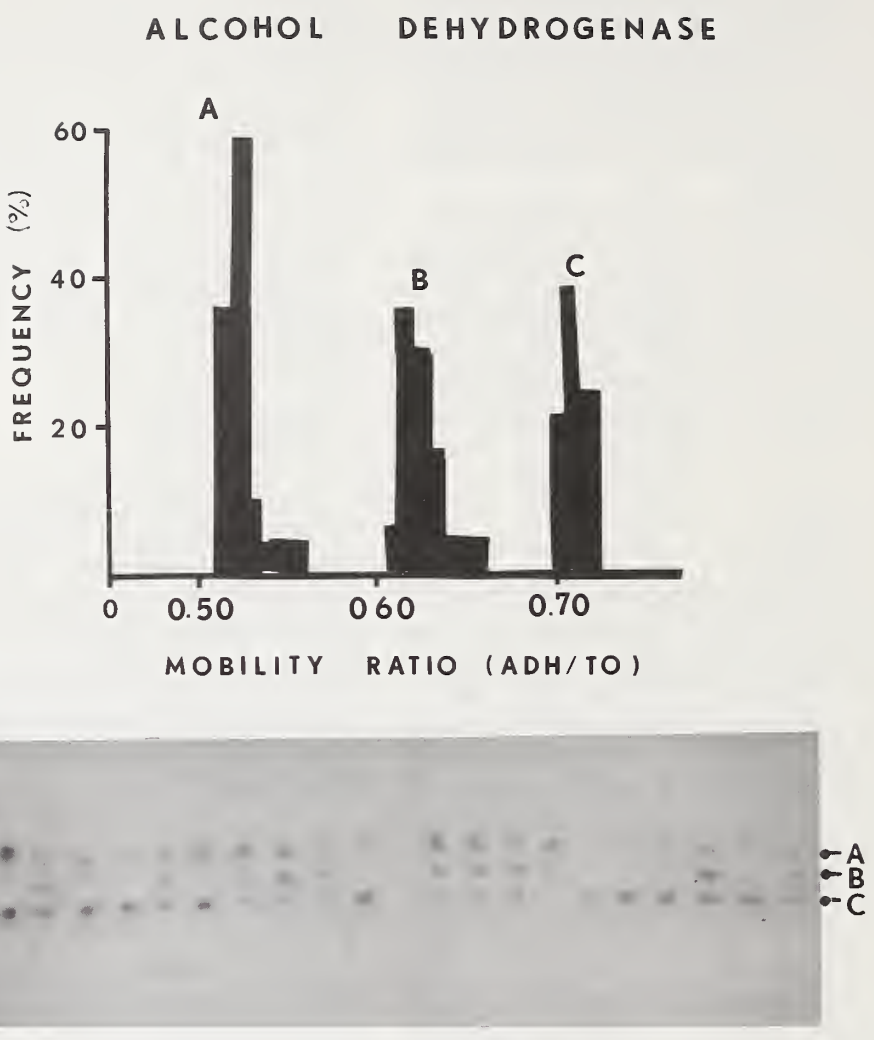
A single, strongly staining zone was associated with the gels treated for G-6-PD activity. This zone, composed of isozyme 0.55, was considered monomorphic (fig. 8).

Glutamate-oxaloacetate transaminase (ALB)

Only one GOT locus was observed in the Florence lab strain, and for practical purposes it was considered monomorphic for isozyme 0.53 (fig. 9). Heterozygosity was detected in only 1 of 108 adults. However, analysis of this locus in other boll weevil strains showed almost 30% heterozygosity in a wild population from South Carolina and almost 50% heterozygosity in a wild population from Texas.

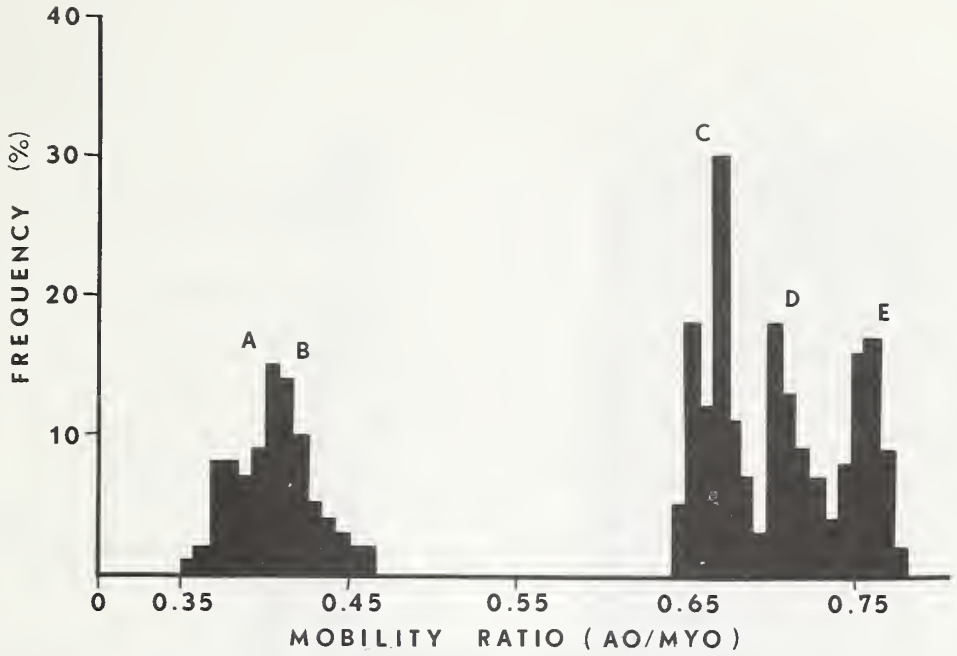
The heterozygote showed a three-banded pattern (isozymes 0.39, 0.47, and 0.53). The appearance of the intermediate (0.47) band indicated proteins of the dimeric type, encoded by two alleles in which the subunits randomly combined.

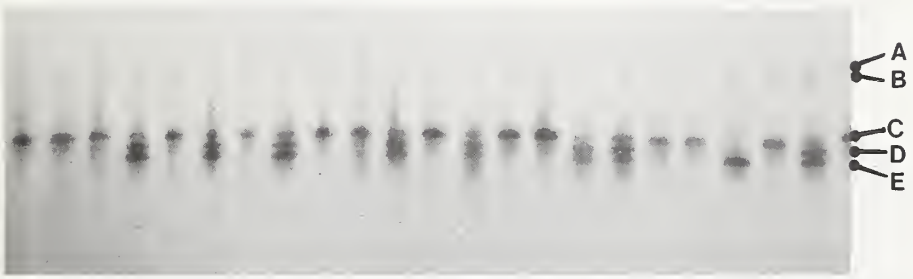
(*Continued on page 45.*)



		MOBILITY RATIO (ADH/TO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	107	0.53	0.004	1.61	0.001	0.52-0.56
B	54	0.63	0.007	1.15	0.001	0.60-0.65
C	92	0.72	0.009	1.79	0.001	0.70-0.72

FIGURE 3.—Distribution of mobility ratios for alcohol dehydrogenase isozymes from 107 adult boll weevils.

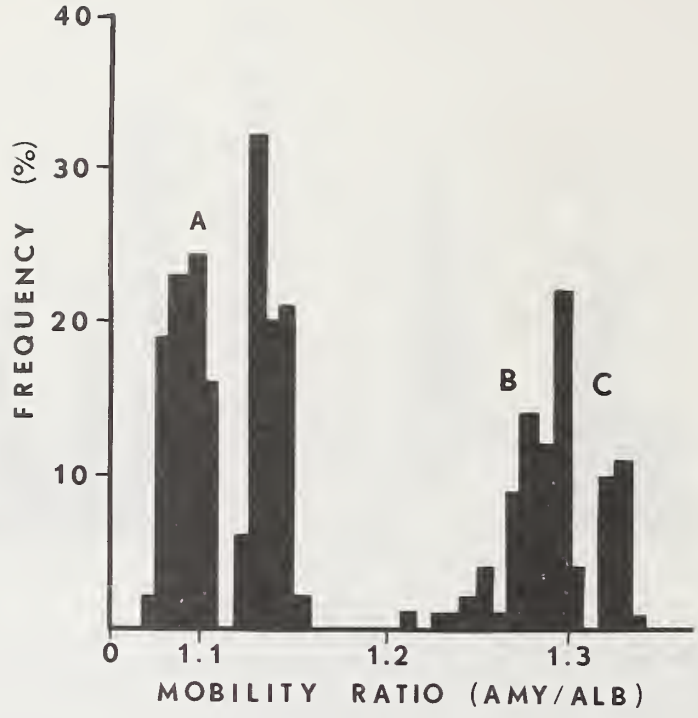




		MOBILITY RATIO (AO/MYO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	45	0.38	0.015	3.89	0.002	0.35-0.40
B	46	0.43	0.016	3.65	0.003	0.40-0.46
C	90	0.67	0.011	1.69	0.001	0.64-0.70
D	50	0.71	0.011	1.49	0.002	0.70-0.74
E	57	0.76	0.011	1.42	0.001	0.74-0.78

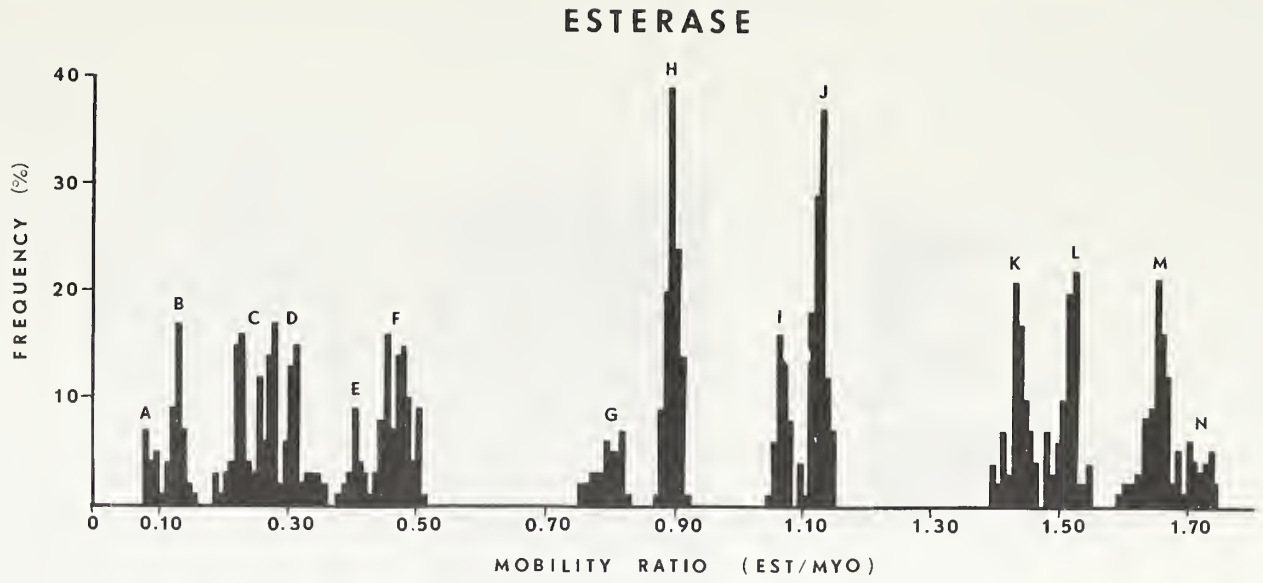
FIGURE 4.—Distribution of mobility ratios for aldehyde oxidase isozymes from 97 adult boll weevils.

AMYLASE



		MOBILITY RATIO (AMY/ALB)				
ISOZYME	N	X	S	CV	SE	RANGE
A	165	1.11	0.017	1.54	0.002	1.07-1.15
B	10	1.25	0.015	1.20	0.005	1.22-1.26
C	84	1.30	0.019	1.44	0.002	1.27-1.34

FIGURE 5.—Distribution of mobility ratios for amylase isozymes from 165 adult boll weevils.



		MOBILITY RATIO (EST/MYO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	17	0.08	0.010	11.81	0.002	0.07-0.10
B	37	0.12	0.008	6.86	0.001	0.11-0.14
C	87	0.24	0.024	10.21	0.003	0.18-0.27
D	62	0.32	0.010	3.27	0.003	0.28-0.35
E	23	0.39	0.012	3.11	0.003	0.36-0.42
F	89	0.48	0.020	4.21	0.002	0.44-0.52
G	37	0.79	0.022	2.76	0.004	0.75-0.83
H	108	0.89	0.009	1.00	0.001	0.87-0.92
I	52	1.06	0.08	0.78	0.001	1.05-1.08
J	108	1.13	0.011	0.94	0.001	1.09-1.15
K	68	1.43	0.015	1.05	0.002	1.39-1.46
L	71	1.50	0.017	1.12	0.002	1.47-1.54
M	86	1.65	0.015	0.91	0.002	1.60-1.69
N	25	1.72	0.016	0.91	0.003	1.69-1.75

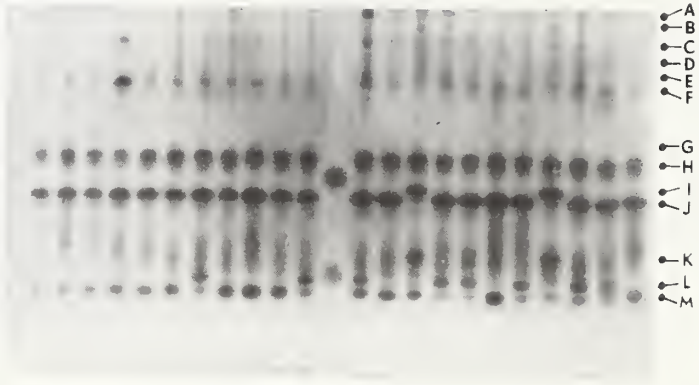
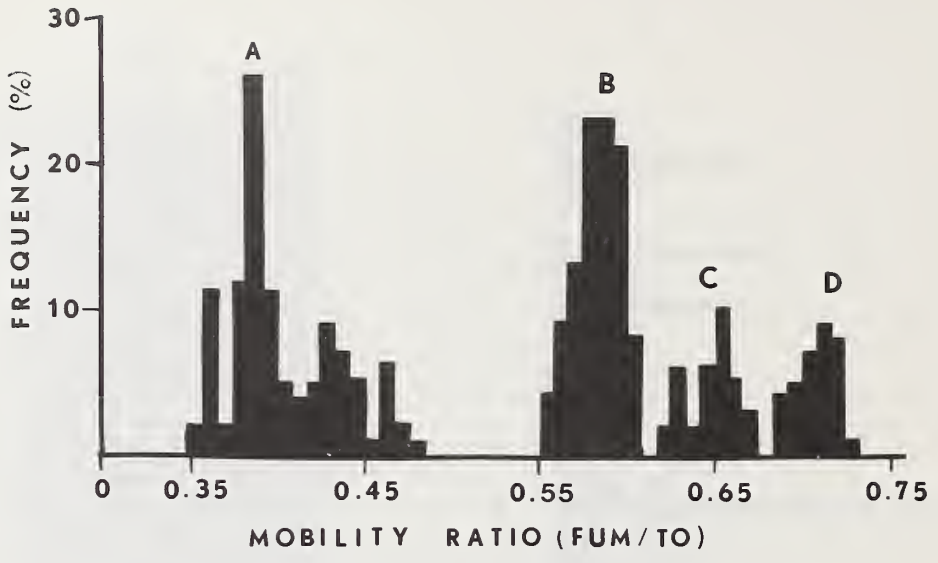
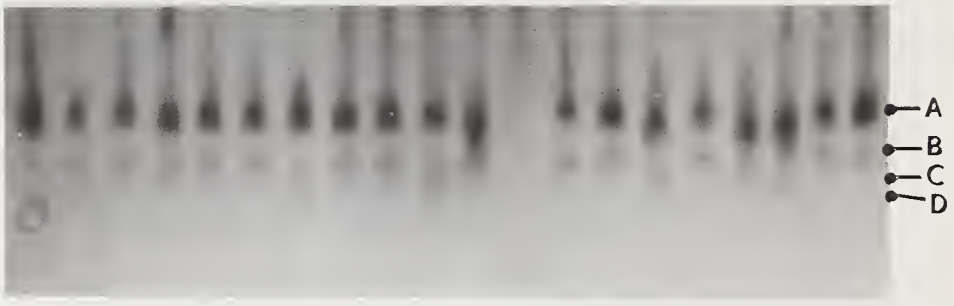


FIGURE 6.—Distribution of mobility ratios for esterase isozymes from 108 adult boll weevils. Esterase 1.72 (N) is not shown.

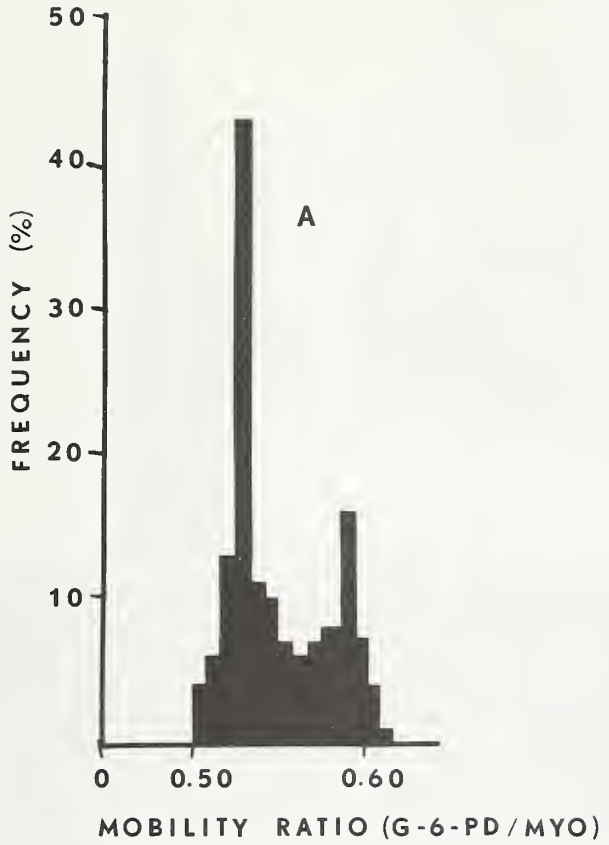
FUMARASE

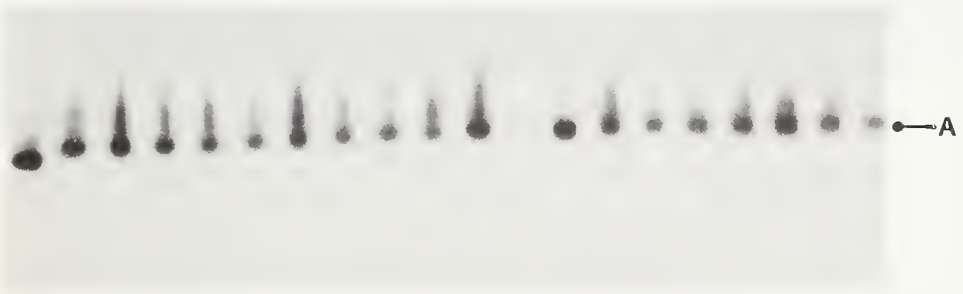




		MOBILITY RATIO (FUM/TO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	109	0.41	0.033	7.96	0.005	0.35-0.46
B	103	0.58	0.014	2.34	0.001	0.55-0.66
C	32	0.65	0.012	1.90	0.002	0.63-0.67
D	26	0.71	0.012	1.63	0.002	0.69-0.73

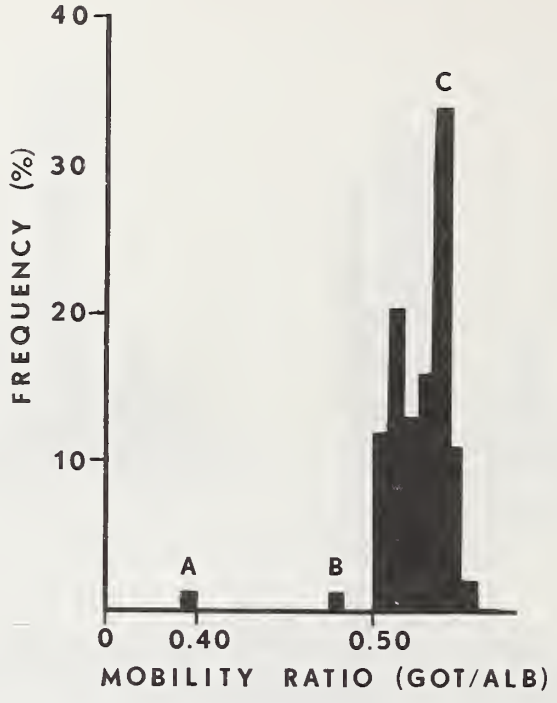
FIGURE 7.—Distribution of mobility ratios for fumarase isozymes from 109 adult boll weevils.

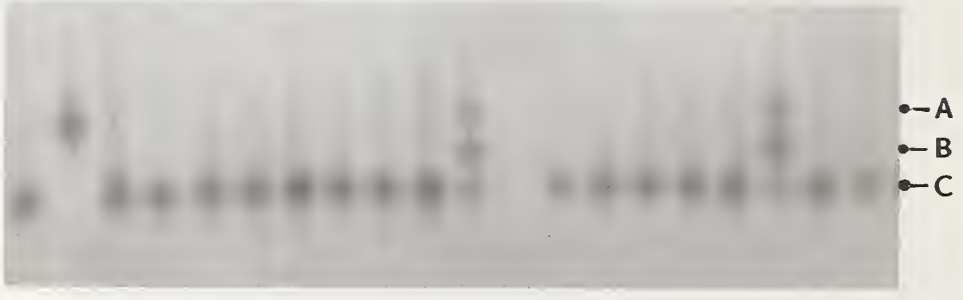




		MOBILITY RATIO (G-6-PD/MYO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	144	0.55	0.024	4.32	0.003	0.51-0.61

FIGURE 8.—Distribution of mobility ratios for glucose-6-phosphate dehydrogenase isozymes from 144 adult boll weevils.

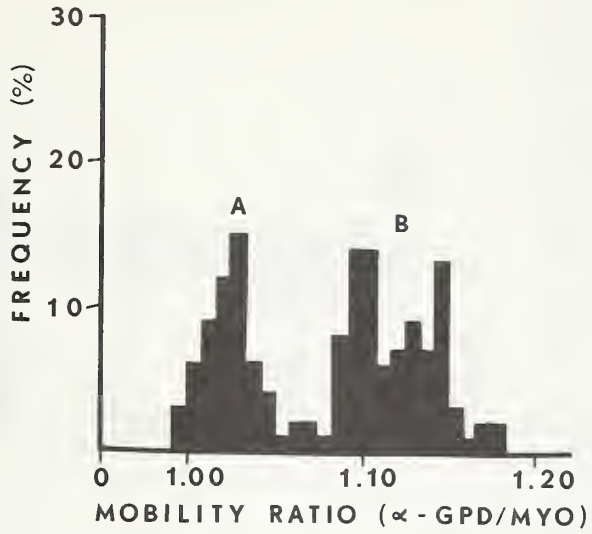


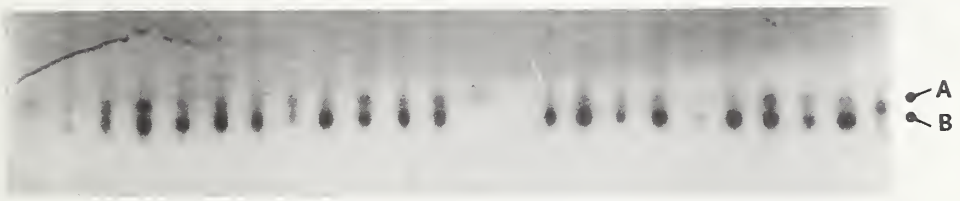


		MOBILITY RATIO (GOT/ALB)				
ISOZYME	N	X	S	CV	SE	RANGE
A	1	0.39	-	-	-	-
B	1	0.47	-	-	-	-
C	108	0.53	0.013	2.45	0.001	0.50-0.55

FIGURE 9.—Distribution of mobility ratios for glutamate-oxaloacetate transaminase isozymes from 108 adult boll weevils. Photograph is from a wild boll weevil population from Brownsville, Tex., to demonstrate the 0.39 homozygote and and the heterozygote.

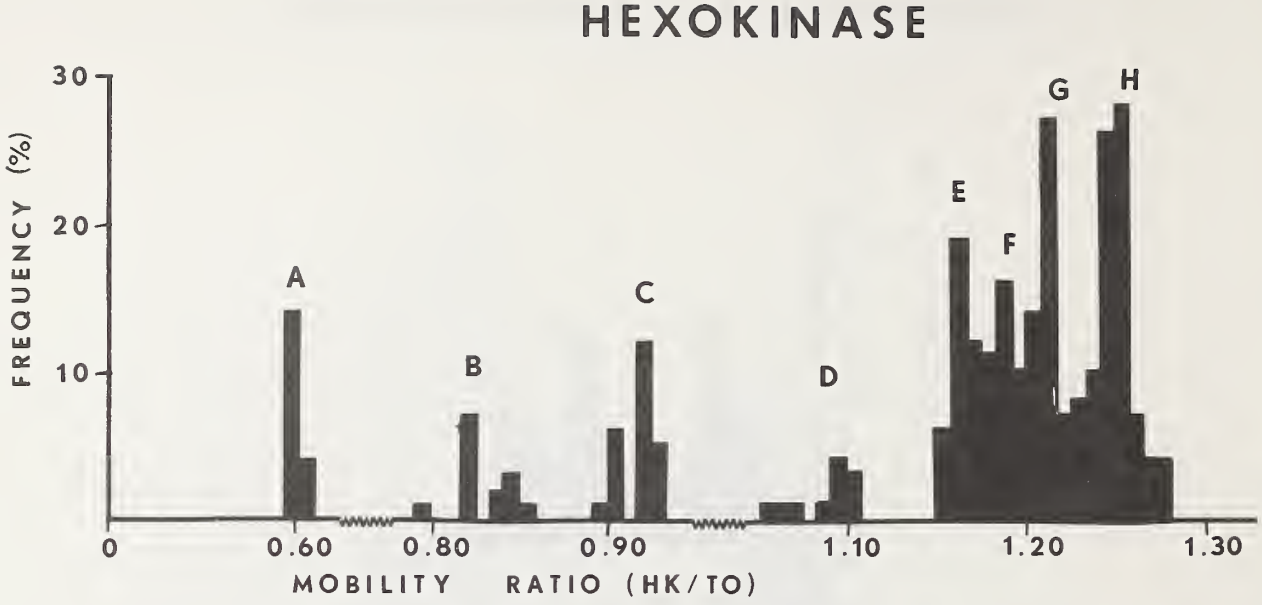
∝-GLYCEROPHOSPHATE DEHYDROGENASE

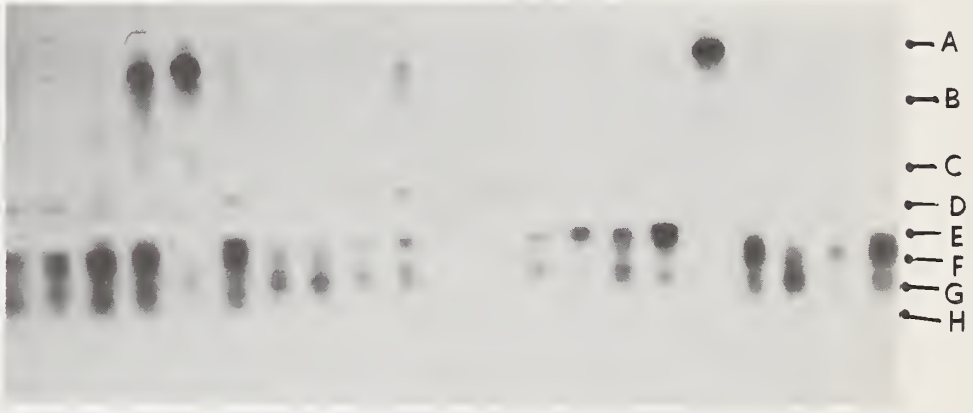




		MOBILITY RATIO (α-GPD/MYO)				
ISOZYME	N	X̄	S	CV	SE	RANGE
A	59	1.03	0.018	1.73	0.002	0.99-1.07
B	90	1.12	0.029	2.57	0.003	1.08-1.19

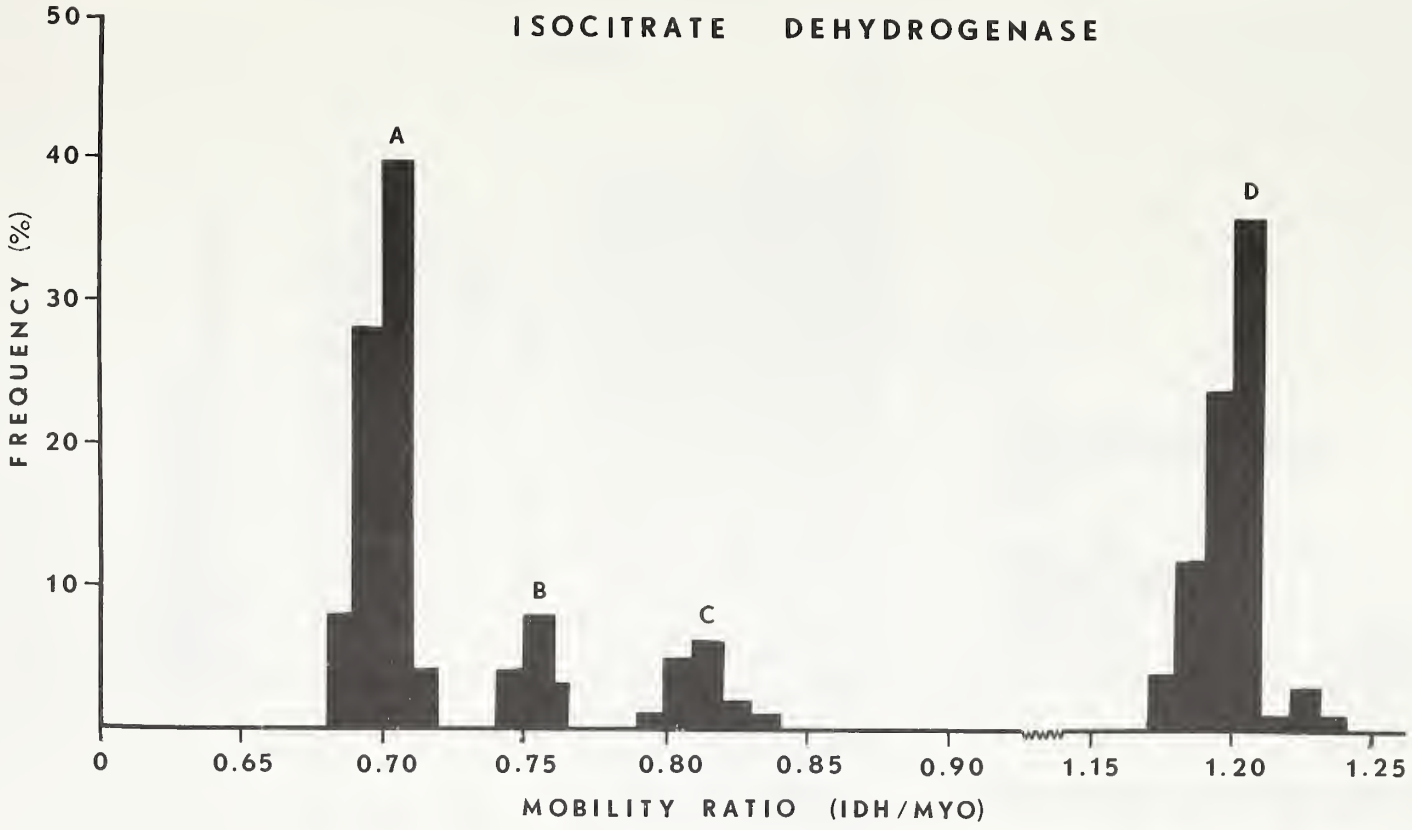
FIGURE 10.—Distribution of mobility ratios for α-glycerophosphate dehydrogenase isozymes from 98 adult boll weevils.





ISOZYME	N	MOBILITY RATIO (HK/TO) X̄	S	CV	SE	RANGE
A	18	0.59	0.004	0.68	0.001	0.59-0.60
B	14	0.83	0.016	1.88	0.004	0.79-0.85
C	22	0.92	0.008	0.92	0.002	0.89-0.93
D	11	1.09	0.007	1.53	0.005	1.06-1.11
E	48	1.17	0.009	0.75	0.001	1.15-1.19
F	40	1.20	0.010	0.81	0.002	1.18-1.22
G	48	1.22	0.016	0.79	0.001	1.20-1.24
H	86	1.26	0.011	0.88	0.001	1.24-1.29

FIGURE 11.—Distribution of mobility ratios for hexokinase isozymes from 86 adult boll weevils.



		MOBILITY RATIO (IDH/MYO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	81	0.69	0.011	1.61	0.001	0.68-0.72
B	15	0.75	0.010	1.33	0.001	0.74-0.77
C	15	0.82	0.012	1.46	0.001	0.79-0.84
D	81	1.20	0.014	1.14	0.002	1.17-1.23

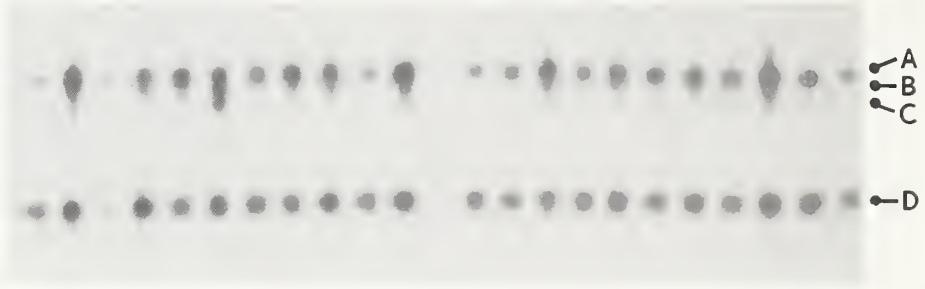
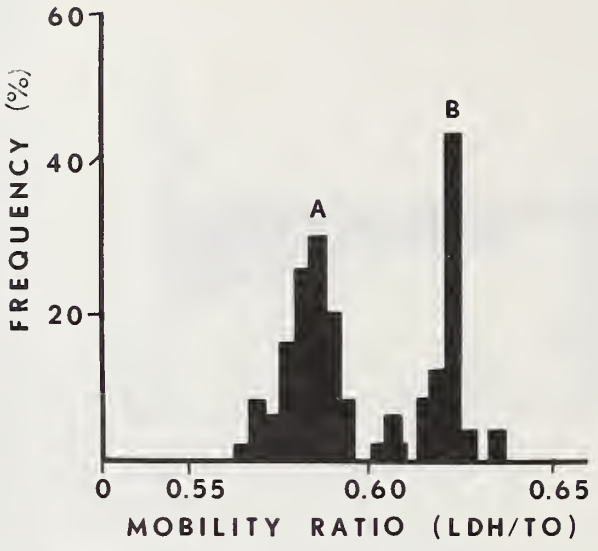
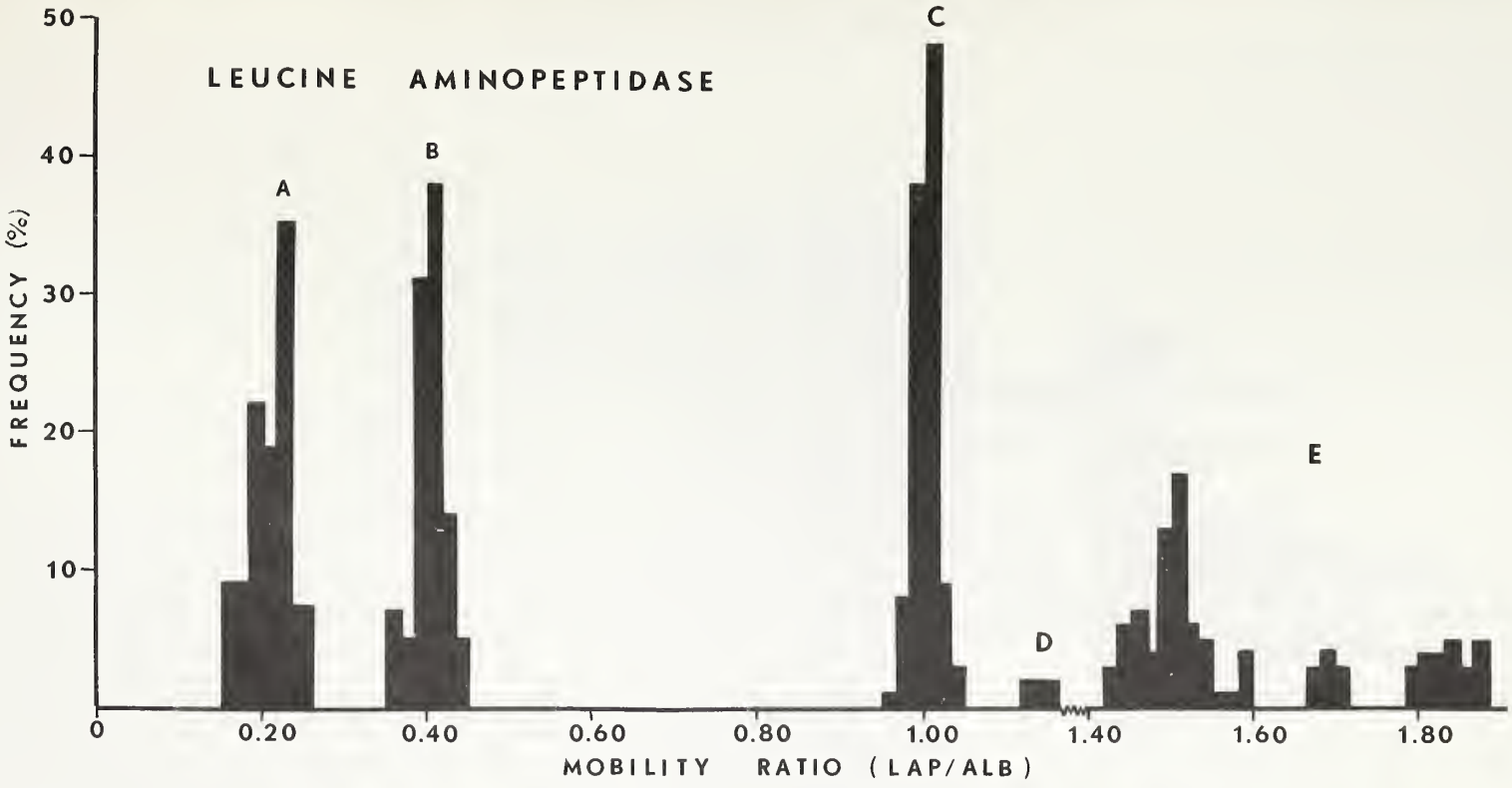


FIGURE 12.—Distribution of mobility ratios for isocitrate dehydrogenase isozymes from 81 adult boll weevils.



		MOBILITY RATIO (LDH/TO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	112	0.58	0.007	1.17	0.001	0.56-0.60
B	82	0.62	0.007	1.06	0.001	0.61-0.64

FIGURE 13.—Distribution of mobility ratios for lactate dehydrogenase isozymes from 112 adult boll weevils.



ISOZYME	N	MOBILITY RATIO (LAP/ALB) X	S	CV	SE	RANGE
A	106	0.21	0.024	11.63	0.002	0.15-0.25
B	106	0.39	0.019	4.74	0.002	0.34-0.44
C	106	1.00	0.014	1.44	0.001	0.96-1.04
D	6	1.14	0.018	1.57	0.007	1.12-1.16
E	102	1.68	0.088	5.39	0.018	1.44-1.88

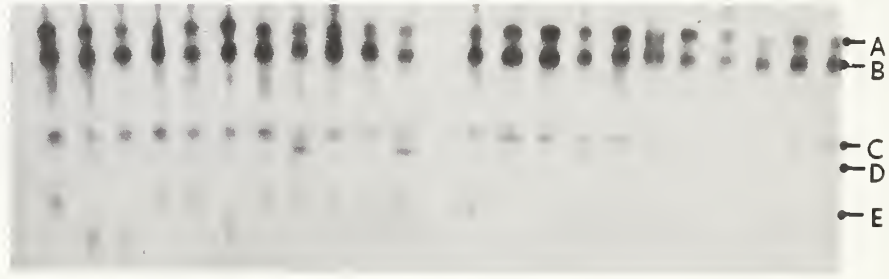
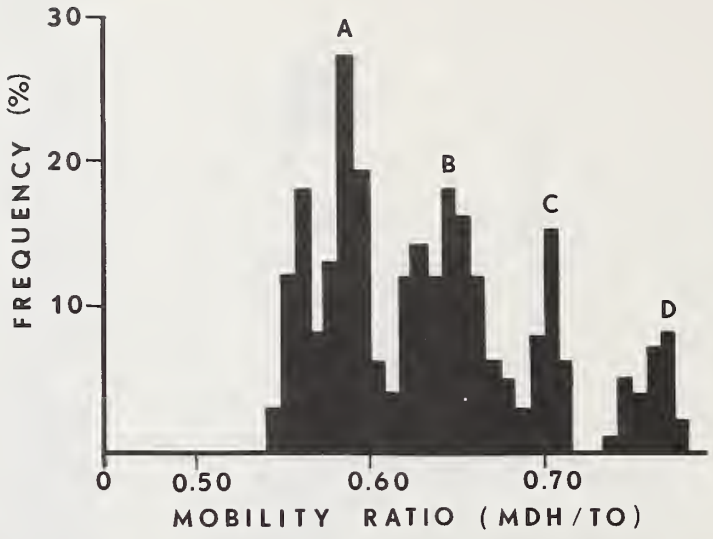


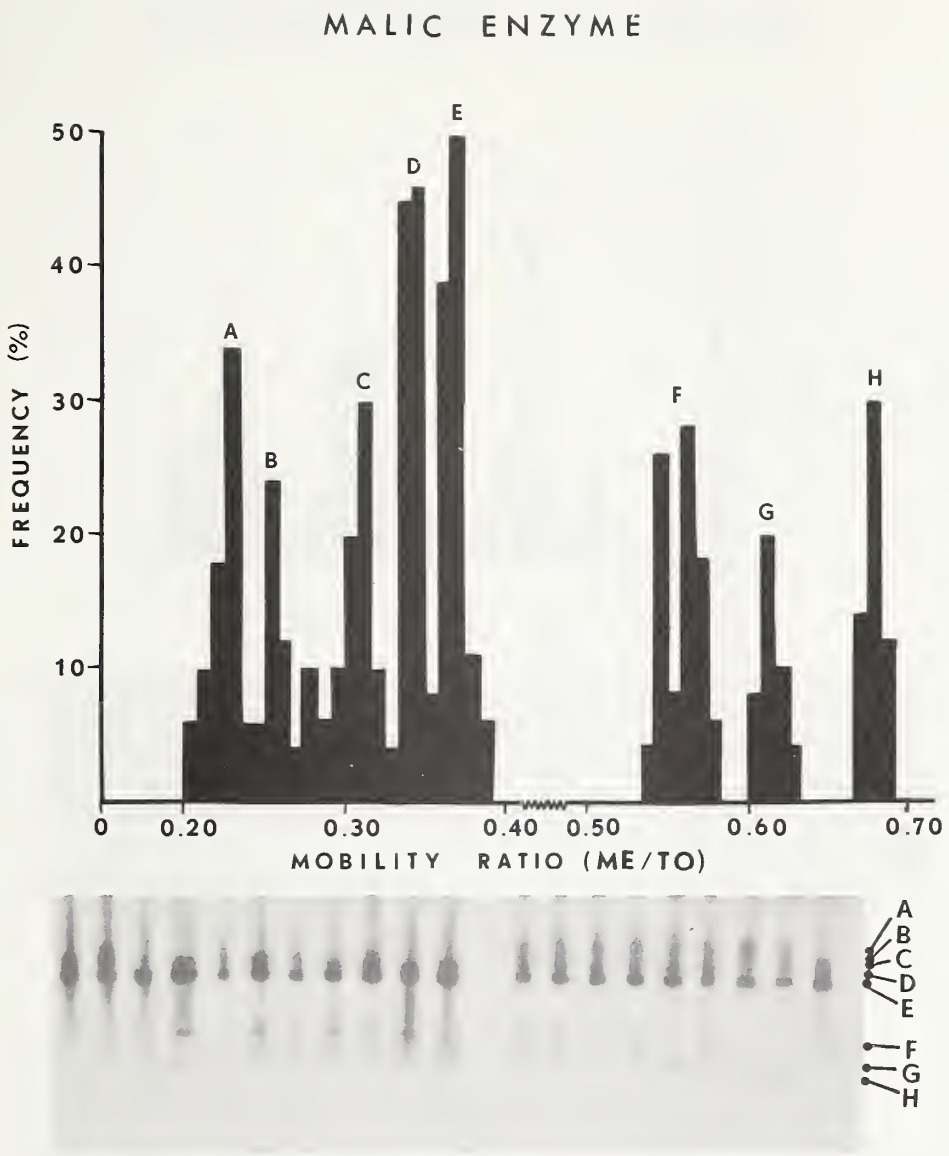
FIGURE 14.—Distribution of mobility ratios for leucine aminopeptidase isozymes from 106 adult boll weevils.

MALATE DEHYDROGENASE



		MOBILITY RATIO (MDH/TO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	106	0.58	0.016	2.77	0.002	0.54-0.60
B	95	0.64	0.016	2.55	0.002	0.61-0.67
C	37	0.70	0.012	1.70	0.002	0.67-0.71
D	27	0.76	0.011	1.47	0.002	0.74-0.78

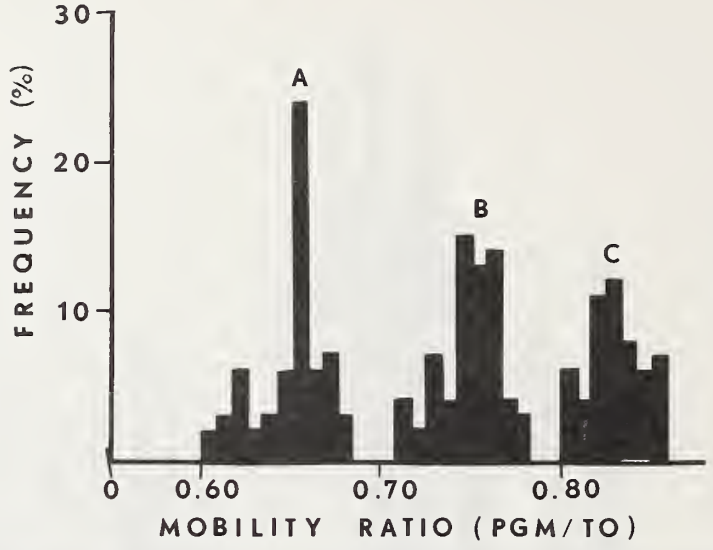
FIGURE 15.—Distribution of mobility ratios for malate dehydrogenase isozymes from 106 adult boll weevils.

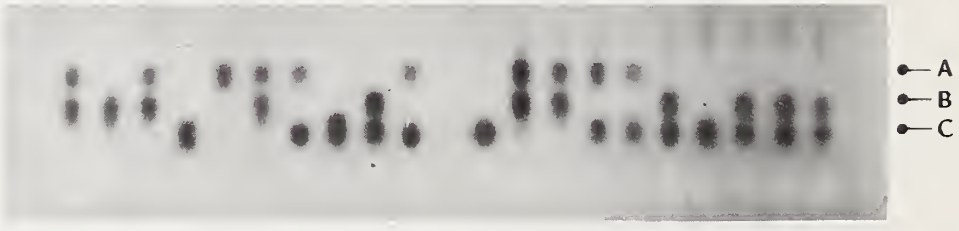


		MOBILITY RATIO (ME/TO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	74	0.22	0.008	3.78	0.001	0.21-0.24
B	62	0.26	0.013	4.90	0.002	0.25-0.29
C	70	0.31	0.007	2.44	0.001	0.29-0.32
D	104	0.35	0.005	1.49	0.001	0.33-0.35
E	104	0.37	0.006	1.55	0.001	0.36-0.39
F	90	0.56	0.012	2.09	0.001	0.54-0.58
G	42	0.62	0.007	1.15	0.001	0.60-0.63
H	56	0.68	0.005	0.79	0.001	0.67-0.69

FIGURE 16.—Distribution of mobility ratios for malic enzyme isozymes from 104 adult boll weevils.

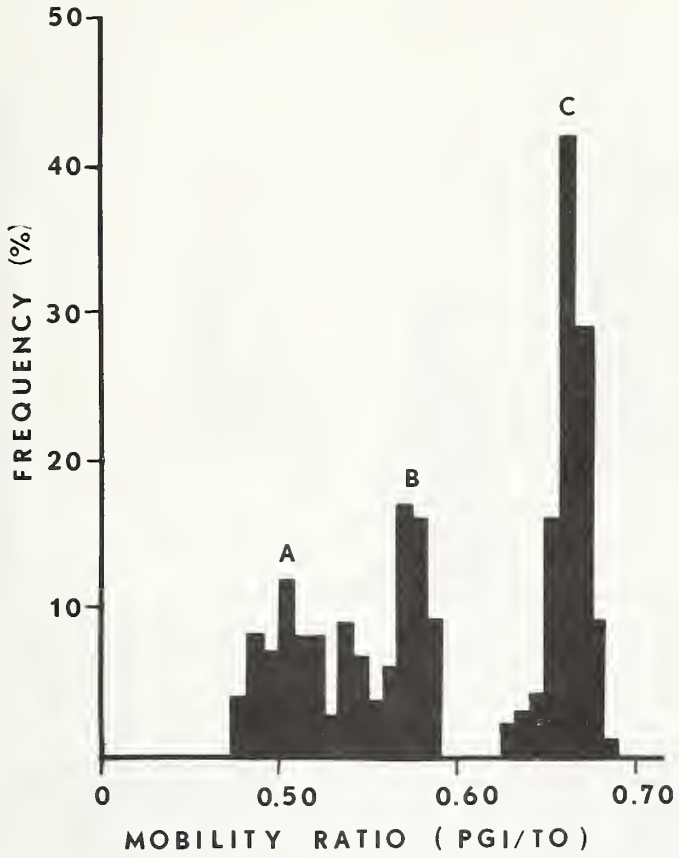
PHOSPHOGLUCOMUTASE





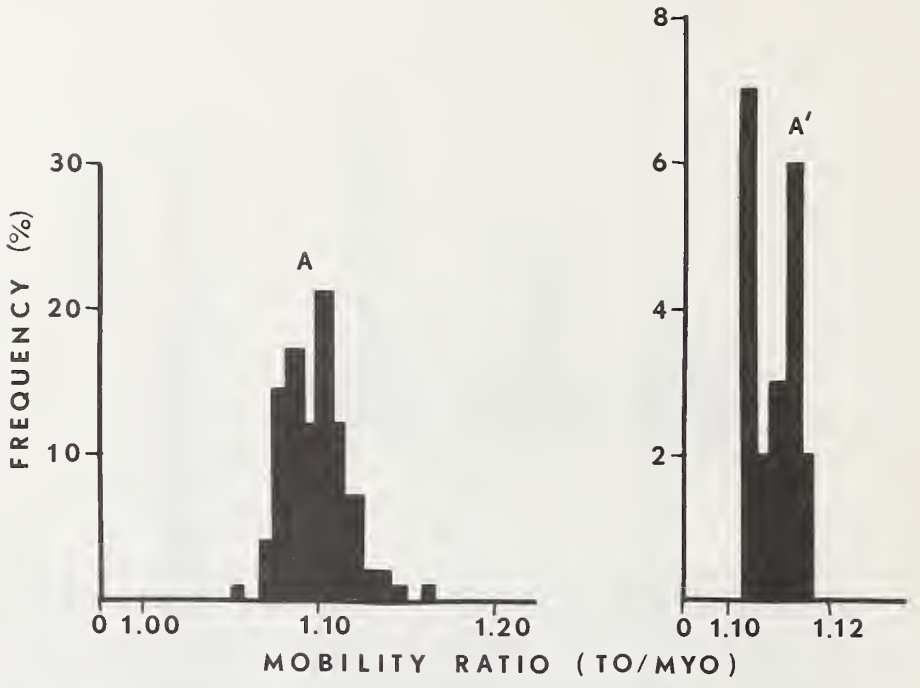
		MOBILITY RATIO (PGM/TO)				
ISOZYME	N	X	S	CV	SE	RATIO
A	62	0.65	0.018	2.77	0.002	0.61-0.68
B	66	0.75	0.016	2.15	0.002	0.71-0.78
C	54	0.83	0.014	1.74	0.002	0.80-0.85

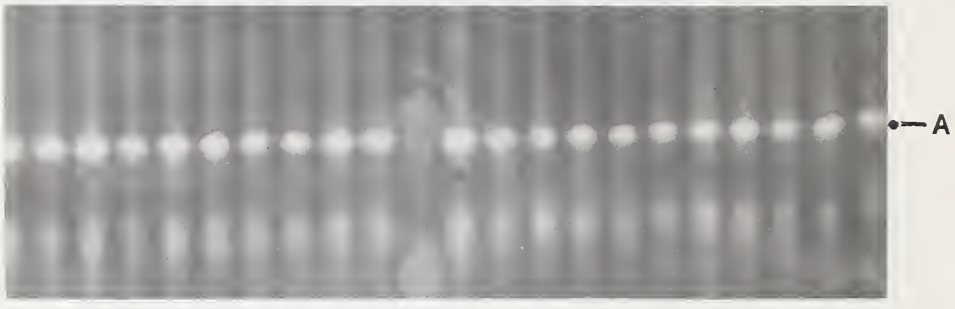
FIGURE 17.—Distribution of mobility ratios for phosphoglucomutase isozymes from 108 adult boll weevils.



		MOBILITY RATIO (PGI/TO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	50	0.50	0.014	2.78	0.002	0.48-0.53
B	68	0.57	0.016	2.82	0.002	0.53-0.59
C	106	0.66	0.010	1.48	0.001	0.63-0.68

FIGURE 18.—Distribution of mobility ratios for phosphoglucose isomerase isozymes from 106 adult boll weevils.





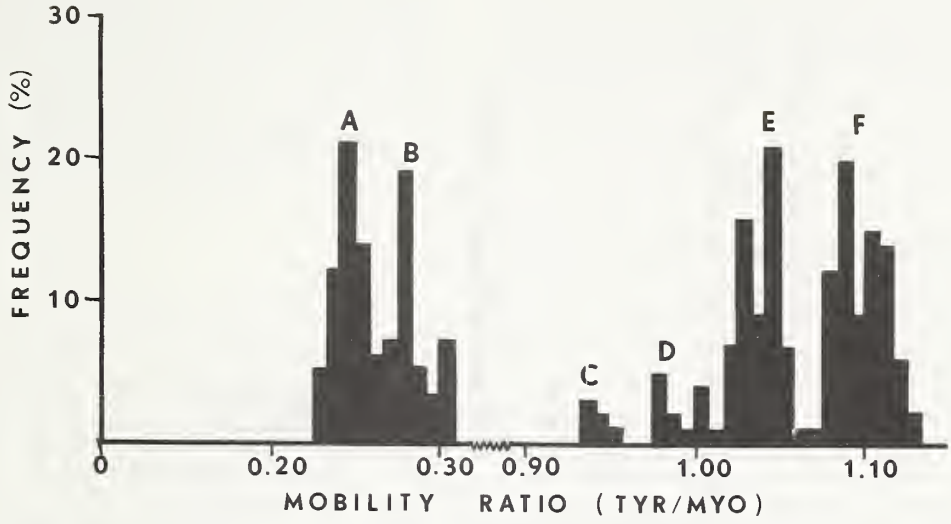
ISOZYME	N 1/	MOBILITY RATIO (TO/MYO) X	S	CV	SE	RANGE
A	100	1.10	0.020	1.86	0.002	1.06-1.17
A^1	20	1.11	0.005	0.48	0.001	1.10-1.12

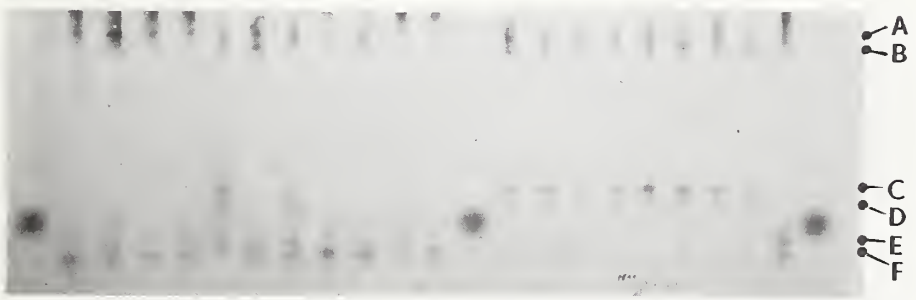
1/ A = 100 GELS, 1 SAMPLE/GEL

A^1= 1 GEL, 20 SAMPLES

FIGURE 19.—Distribution of mobility ratios for tetrazolium oxidase isozymes from 100 adult boll weevils.

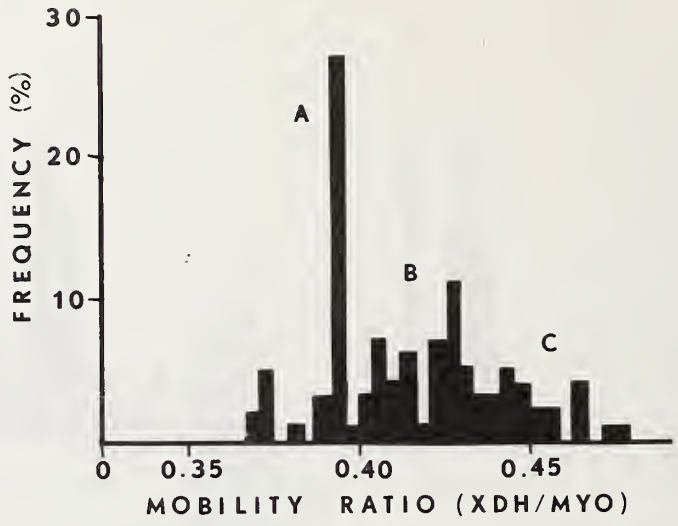
TYROSINASE

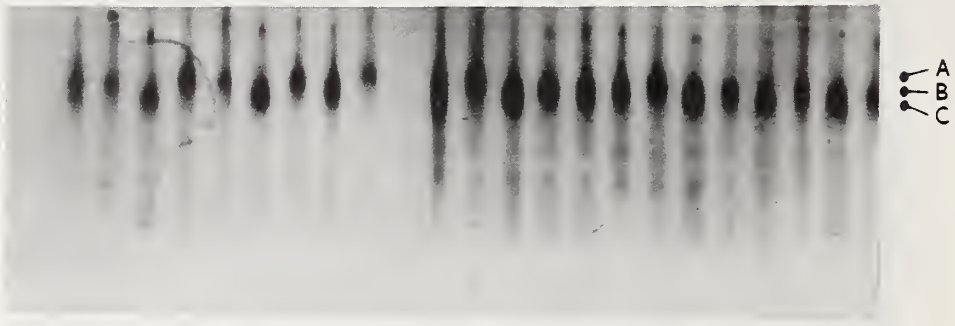




		MOBILITY RATIO (TYR/MYO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	57	0.25	0.009	3.52	0.001	0.23-0.27
B	41	0.28	0.011	3.80	0.002	0.27-0.30
C	6	0.94	0.008	0.84	0.003	0.93-0.95
D	13	0.99	0.011	1.11	0.003	0.97-1.01
E	62	1.04	0.011	1.07	0.001	1.02-1.06
F	78	1.10	0.014	1.31	0.002	1.07-1.13

FIGURE 20.—Distribution of mobility ratios for tyrosinase isozymes from 94 adult boll weevils.





		MOBILITY RATIO (XDH/MYO)				
ISOZYME	N	X	S	CV	SE	RANGE
A	37	0.39	0.008	2.06	0.001	0.37-0.40
B	45	0.42	0.011	2.57	0.002	0.40-0.43
C	23	0.45	0.012	2.68	0.003	0.43-0.47

FIGURE 21.—Distribution of mobility ratios for xanthine dehydrogenase isozymes from 106 adult boll weevils.

α-Glycerophosphate dehydrogenase (MYO)

A single zone of α-GPD activity was observed in the Florence lab strain. Isozyme patterns of either one band (1.03 or 1.12) or two bands (1.03 and 1.12) were observed in individual boll weevils (fig. 10). Thus, a polymorphic locus was indicated. This interpretation conformed to the Hardy-Weinberg equilibrium criteria set forth earlier (table 10).

Hexokinase (TO)

Three zones of HK activity were observed (fig. 11). However, the two slowest migrating zones appeared sporadically and were usually light in stain intensity when present on the gels. Both of these zones were polymorphic in nature, but interpretation was not attempted. The third zone of HK activity gave intense band staining and was apparently polymorphic, but genetic mechanisms were not determined. Inheritance studies may resolve some of the difficulties experienced with this system, but different electrophoretic conditions or sample handling appear necessary to obtain reliable data.

Isocitrate dehydrogenase (MYO)

Two zones of strong activity were present in IDH-treated gels (fig. 12). The slowest migrating zone contained three isozymes with Rmyo ratios of 0.69, 0.75, and 0.82. Individual boll weevils showed either the 0.69 or 0.82 bands or a three-banded pattern composed of all three isozymes. Genetic interpretation presumed that these patterns represented a single locus composed of alleles 0.69 and 0.82. It was also inferred that the appearance of three bands was characteristic of a dimeric enzyme. The fastest migrating zone of IDH activity resulted in a single isozyme (1.20). This was interpreted as being representative of a monomorphic locus.

Lactate dehydrogenase (TO)

Only two isozymes were consistently present in gels treated for LDH activity in this boll weevil strain (fig. 13). Their mobility ratios were 0.58 and 0.62, respectively. In appearance, isozyme 0.58 was much denser than 0.62 and was always present. When these isozymes were treated as products of a single locus with two alleles, chi-square analysis showed no Hardy-Weinberg equilibrium. Other apparent isozymes were present in some insects but were infrequent. Further study, for both proper electrophoretic conditions and inheritance, is needed for this locus.

Leucine aminopeptidase (ALB)

Five major LAP isozymes were evident in the Florence lab strain (fig. 14). These were tentatively divided into four loci. Isozymes 0.21 and 0.39 were considered representative of monomorphic loci, since each boll weevil possessed both bands. Isozymes 1.00 and 1.14, which occurred either singly or in combination, were considered as constituting a third (polymorphic) locus. Isozyme 1.68, which showed considerable variation in migration, was considered a fourth (monomorphic) locus.

Malate dehydrogenase (TO)

Four bands of MDH activity were apparent (fig. 15). Interpretation of the banding patterns suggested that isozymes 0.58 and 0.70 were alleles, whereas bands 0.64 and 0.76 were conformers of the other pair. The latter two isozymes were not always observed in every individual and were usually stained much lighter than their respective allelic partners. Individuals scored as homozygotes usually showed two bands—the strongly reacting 0.58 band and its weaker-reacting 0.64 partner or the 0.70 band and its 0.76 partner. In some cases, only 0.58 or 0.70 isozymes were apparent. Individuals that were scored as heterozygotes showed three distinct bands with mobilities of 0.58, 0.64, and 0.70. Also, a fourth weak staining band, with mobility of 0.76, was usually present. This would correspond to the conformer of the 0.70 allele. These observations suggested that MDH was represented by a single locus and that each protein was of the dimeric type.

Malic enzyme (TO)

Two distinct zones of ME activity were observed (fig. 16). The slowest migrating area was densely stained and was composed of up to five isozymes.

However, most of these appeared to be artifacts and were not clearly defined because of background smearing. Only isozyme 0.35 was consistently clear, and it was considered a monomorphic locus since it was present in all individuals. The second area of ME activity contained isozymes 0.56, 0.62, and 0.68. These isozymes stained light to medium in intensity. This zone conformed to a genetic interpretation of being a single locus whose alleles produced a single band for the homozygotes and three bands, characteristic of a dimeric enzyme, for the heterozygotes.

Phosphoglucomutase (TO)

The Florence lab strain had one PGM locus consisting of three alleles, 0.65, 0.75, and 0.83. All three stained moderately to intensely, as shown in figure 17. Phenotypically, six patterns were easy to distinguish. Homozygotes exhibited single bands, whereas heterozygotes exhibited two bands, which is characteristic of monomeric proteins. Chi-square evaluation for Hardy-Weinberg proportions yielded a P value greater than 80% (table 10).

Phosphoglucose isomerase (TO)

Two zones contained PGI activity (fig. 18). The slowest migrating zone, isozymes 0.50 and 0.57, was tentatively identified as one locus and was considered polymorphic. However, from its appearance (bands smeared and not distinct), the possibility of artifactual products remains. Moreover, chi-square evaluation for Hardy-Weinberg equilibrium did yield an acceptable probability value of about 10%. Thus, this zone was only tentatively accepted as a polymorphic locus. The second and fastest migrating zone, represented by isozyme 0.66, was considered a monomorphic locus, since it was present in all individuals tested.

Tetrazolium oxidase (MYO)

The Florence lab boll weevil strain, as with all other boll weevil strains tested, had an invariant TO isozyme at Rmyo 1.10, as discussed earlier (fig. 19). This was considered a monomorphic locus. Two other areas of TO activity were present, but for the most part, they were difficult to score and were not included.

Tyrosinase (MYO)

Six isozymes containing TYR activity were detected (fig. 20). These were tentatively grouped into three loci, each composed to two alleles. The first was dark-staining with low mobility. With the development techniques used, these two bands (0.25 and 0.28) usually developed fully within 60 minutes. The second locus (isozymes 0.94 and 0.99) and third locus (isozymes 1.04 and 1.10) required 2 to 3 hours' development time to produce readable gels. The second locus was of low frequency in the Florence lab strain and was omitted from further study. This locus was fairly common in other boll weevil strains. Table 10 lists the gene frequency data for the first and third loci.

Xanthine dehydrogenase (MYO)

One polymorphic XDH locus was operating in the Florence lab strain. It was composed of two moderately staining alleles, 0.39 and 0.45 (fig. 21). Both heterozygotes and homozygotes produced bands that were broad and diffuse. All three phenotypes were difficult to score because of their diffuse appearance. Distinct bands were rarely seen in the heterozygote, and its presence was detected by an Rmyo value that lay between those of the homozygotes, as shown in figure 21, peak B.

SUMMARY AND CONCLUSIONS

In these tests of 24 enzyme systems, 105 isozymes were detected in the Florence lab strain of boll weevil. Of these isozymes, 67 were assigned either allele or conformer status, as explained in the text and in table 10; 38 were not classified.

The assigned isozymes represented 35 tentative loci, of which 60% were polymorphic and 40% were monomorphic. The average total observed frequency of heterozygotes per locus ($\overline{H}$) was 0.236, with a standard error of 0.041. At the 5% significance level by chi-square evaluation, all of the 21 presumably polymorphic loci were accepted as being

representative of a randomly mating population according to the Hardy-Weinberg equilibrium rule.

The 0.236 value for the average frequency of heterozygotes per locus ($\overline{H}$) was surprising in light of the average heterozygosity of about 0.150 for most other insect species studied and the 0.060 average value for 68 vertebrate species (Ayala 1978). It is interesting, though, that the greatest degree of genetic polymorphism reported among insects is that of a coleopteran insect having an $\overline{H}$ value of 0.309 (Ayala 1978) and that this insect, *Otiorhynchus scaber* (L.), belongs to the same family as the boll weevil.

However, whether the 0.236 value is valid depends upon the accuracy of assigning the correct allelic status to the observed isozymes. Some of the assignments made in this study may prove incorrect once formal inheritance studies are completed. Moreover, many of the 38 isozymes not considered also appeared to be polymorphic in nature. Once these are classified, the average heterozygosity per locus may well exceed the 0.236 value.

Although most of the methodologies used in this study are suitable for general screening of boll weevil populations to determine whether differences exist, some are not suitable for making valid decisions as to the nature of the isozymes (e.g., AK, HK, LDH, and some EST). We conclude that further testing is needed for these systems to determine whether the isozymes represent size, charge, or conformational isozymes; whether they are products of protein-protein or protein-buffer interaction; or whether they are indeed gene products.

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